Tamas Fulop, Claudio Franceschi
Katsuiku Hirokawa, Graham Pawelec
Editors

Handbook on Immunosenescence

Basic Understanding and Clinical Applications

Springer

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Editors

Tamas Fulop
University of Sherbrooke, Quebec, Canada

Claudio Franceschi
University of Bologna, Bologna, Italy

Katsuiku Hirokawa
Institute for Health and Life Sciences, Tokyo, Japan

Graham Pawelec
University of Tübingen, Tübingen, Germany

Springer

Editors
Tamas Fulop
Research Center on Aging
Division of Geriatrics
Dept. of Medicine, Faculty of Medicine
1036 Rue Belvedere
Sherbrooke J1H 4C4
Canada
tamas.fulop@usherbrooke.ca

Claudio Franceschi
CIG Interdepartmental Center
"L. Galvani"
University of Bologna
Department of Experimental Pathalogy
Via San Giacomo 12
40126 Bologna
Italy
claudio.franceschi@unibo.it

Katsuiku Hirokawa
Institute for Health and Life Sciences
4-6-22 Kohinato
Tokyo
Bunkyo-ku
112-0006 Japan

Graham Pawelec
University of Tübingen
ZMF - Zentrum Med. Forschung
Abt. Transplant./ Immunologie
Waldhörnlestr. 22
72072 Tübingen
Germany
graham.pavelec@uni-tuebingen.de

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Preface

What is Immunosenescence?

The number of elderly people is steadily increasing in most countries. Concomitantly, the number of age-related diseases is unfortunately also increasing. One of the leading causes of death in the very elderly is infection, with cardio-vascular diseases and cancer less prevalent than in younger elderly. All three major pathologies are to some extent related to the immune system due to its well-known but still imperfectly investigated deregulation during aging.

Thus, the large amount of data accumulated during the last decade or more has allowed a better but still incomplete understanding of all the complex alterations affecting the immune system with aging. Although we do not know everything, we feel that it is important for the scientific community to become more acquainted with the corpus of knowledge recently generated in this domain, presented in a manner providing a critical evaluation of the current status of research. Many accepted ideas have changed during the last decade, such as the effect of aging on the innate immune system, antigen presentation, the cytokine imbalance and low grade inflammation. If not exactly a paradigm shift, the time seems ripe to present this critical evaluation and update of the state-of-the-art in these different areas. We perceive a great need to assemble this current knowledge in one volume by collecting contributions from the most eminent researchers in the field from all around the world. In this way, we aim to facilitate a synthesis of the different aspects of the disparate disciplines in ageing research to focus on immunosenescence for the first time (basic and clinical, molecular, cellular, biochemical, genetics). We hope this multidisciplinary approach from the aging, immunity and inflammation community will also be important for future innovative research in this domain.

Thus, this book will have as its main themes Aging, Immunity and Inflammation, with an emphasis on studies in humans. However, as data are not always available in this species, work in experimental animals will be also treated as appropriate. A large number of colleagues responded enthusiastically to our proposal and contributed with very high quality chapters. We begin with a description of Methods and models for studying immunosenescence. We continue with Cellular immunosenescence, treating most specifically T cells, B cells, neutrophils, antigen presenting cells

and NK cells. We then proceed to mechanisms. In this context, receptor signaling, the role of mitochondrial activity, the proteasome, cytokine status and the neuro-endocrine-immune netweork are treated. The important but very challenging area of the Clinical relevance of immunosenescence for disease states is covered next by the individual treatment of infections, autoimmunity, cancer, metabolic syndrome, neurodegeneration and frailty. Finally, and even more challengingly, the last part of the book is devoted to possibilities for eventual intervention and modulation. We particularly emphasise nutritional aspects, lipids and experimental interventions. In this way we feel that we cover the whole range of areas from models, through basic molecular mechanisms to the clinical relevance and finally eventual modulation.

One of the main objectives of this book is to present in a systematic way our current knowledge in the field of the immunology related to aging. So do we now know what immunosenescence is? It is still difficult at answer this question, but we hope even the most specialist investigator in the field will find concepts and ideas within the book which will help him or her to approach an answer to this important question more closely than before. We would therefore sincerely like to hope that we have created an authoritative, innovative and thought-provoking book dedicated for the first time to this topic alone. We also like to hope that this volume will help to attract a new generation of researchers to the field of immunosenescence as an expanding and vital research arena.

Tamas Fulop — Quebec, Canada
Claudio Franceschi — Bologna, Italy
Katsuiku Hirokawa — Tokyo, Japan
Graham Pawelec — Tübingen, Germany

Contents

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Cellular Immunosenescence - NK and NKT Cells

Contributors

A.I. Yashin
Center for Population Health and Aging, Duke University, Durham, USA

A.M. Robert
Université paris 5. Laboratoire de Recherche Ophtalmologique., 1 place du Parvis Notre Dame, 75181 Paris cedex 04, France

Agnieszka Brzezińska
Molecular Bases of Aging Laboratory, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Pasteura 3, Warsaw Poland

Alan D. Roberts
Trudeau Institute, Saranac Lake, NY 12983, USA

Alessia Donnini
Laboratory of Tumour Immunology, INRCA Res. Dept., Via Birarelli 8, 60121 Ancona, Italy

Alexey A. Romanyukha
Institute of Numerical Mathematics, Russian Academy of Sciences, Moscow, Russia

Anders Wikby
Department of Natural Science and Biomedicine, School of Health Sciences, Jönköping University, Box 1026, 551 11 Jönköping, Sweden

Andrea Cossarizza
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Andrea Rossmann
Division of Experimental Pathophysiology and Immunology, Laboratory of Autoimmunity, Biocenter, Innsbruck Medical University, Fritz-Pregl-Strasse 3, A-6020-Innsbruck, Austria

Anis Larbi
Center for Medical Research, Section for Transplant-Immunology and Immuno-Hematology, Tuebingen Aging and Tumor Immunology group, University of Tuebingen Medical School, Waldhörnlestr. 22, D-72072 Tübingen, Germany

Ankmalika Gupta
Division of Basic and Clinical Immunology, University of California, Irvine, California

Ann Chidgey
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Anna C. Phillips
School of Sport and Exercise Science, Birmingham University Medical School, Birmingham B15 2TT, UK

Anna Lang
Vaccine and Gene Therapy Institute, Department of Molecular Microbiology and Immunology and the Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006, USA

Anne Fletcher
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Anshu Agrawal
Division of Basic and Clinical Immunology, University of California, Irvine, CA 92697, USA

Antonio Celada
Institute for Research in Biomedicine-University of Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain

Arianna Smorlesi
Laboratory of Tumour Immunology, INRCA Res. Dept., Via Birarelli 8, 60121 Ancona, Italy

Arie Ben Yehuda
University Hospital Kerem, The Department of Medicine at the Hadassah Ein, Jerusalem, Isreal

Barry W. Ritz
Drexel University, Department of Bioscience & Biotechnology, 118 Stratton Hall, 32nd and Chestnut Streets, Philadelphia, PA 19104, USA

Beatriz Sánchez-Correa
Immunology Unit, Department of Physiology, University of Extremadura, Cáceres, Spain

Bert E. Johansson
Innovation Sciences, Armonk, NY 10504, USA

Bertrand Friguet
Laboratoire de Biologie Cellulaire du Vieillissement, UMR 7079, Université Pierre et Marie Curie, 4 Place Jussieu, 75005 Paris, France

Blair Henderson
Division of Experimental Pathophysiology and Immunology, Laboratory of Autoimmunity, Biocenter, Innsbruck Medical University, Fritz-Pregl-Strasse 3, A-6020-Innsbruck, Austria

Bonnie B. Blomberg
Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101, USA

Boo Johansson
Institute of Gerontology, School of Health Sciences, Jönköping University, Box 1026, 551 11 Jönköping, Sweden, and Department of Psychology, Göteborg University, Box 500, 405 30 Göteborg, Sweden

Bruno Lesourd
EA 2431, Faculté de Médecine, 28 Place Henri Dunant, 63001 Clermont-Ferrand and Hôpital Nord du CHU de Clermont-Ferrand, BP 36, 63118 Cebazat, France

Claudio Franceschi
Department of Experimental Pathology, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy; CIG-Interdepartmental Center “L. Galvani”, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy

Calogero Caruso
Gruppo di Studio sull’Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Carl Fortin
Clinical research Center, Graduate Immunology Program, Division of Pulmonology, Department of Medicine, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada

Carlos Sebastián
Institute for Research in Biomedicine-University of Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain

Carmela Rita Balistreri
Gruppo di Studio sull’Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Christina Mayerl
Division of Experimental Pathophysiology and Immunology, Laboratory of Autoimmunity, Biocenter, Innsbruck Medical University, Fritz-Pregl-Strasse 3, A-6020-Innsbruck, Austria

Christian R. Gomez
The Burn and Shock Trauma Institute and the Immunology and Aging Program; Department of Surgery; Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA; Facultad de Ciencias de la Salud, Universidad Diego Portales, Ejército 141, Santiago, Chile

Christopher A. Jolly
Division of Nutritional Sciences, The University of Texas, Austin, TX 78712, USA

Clarice Luz
LabVitrus, Rua Garibaldi, 659/502, Porto Alegre, RS 90035-050, Brazil

Claude Sportès
Experimental Transplantation & Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, MD, USA

Claudia Gravekamp
California Pacific Medical Center Research Institute, 475 Brannan Street, San Francisco, CA 94107, USA

Cornelia M. Weyand
Kathleen B. and Mason I. Lowance Center for Human Immunology, Department of Medicine, Emory University School of Medicine, Room 1003 Woodruff Memorial Research Building, 101 Woodruff Circle, Atlanta, GA, USA

Corona Alonso
Department of Immunology, Reina Sofia University Hospital, University of Córdoba, Corodoba, Spain

Cristina Bonorino
Faculdade de Biociências and Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga 6690, 2º andar. P.O. Box 1429. Porto Alegre, RS 90.610-000, Brazil

Cristina Gatti
Laboratory of Tumour Immunology, INRCA Res. Dept., Via Birarelli 8, 60121 Ancona, Italy

Cristina M. Moriguchi Jeckel
Faculdade de Farmácia, PUCRS, Av. Ipiranga, 6681. Porto Alegre, RS 90619-900, Brazil

Daniela Frasca
Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101, USA; Graduate School of Cell Biology and Development, University of Rome La Sapienza, Rome, Italy

Daniela Monti
Department of Oncology and Experimental Pathology, University of Florence, Via Morgagni 50, Florence, Italy

David Bryder
Stem Cell Aging, Department of Experimental Medical Science, BMC D14, Lund University, 221 84 Lund, Sweden

David L. Lamar
Kathleen B. and Mason I. Lowance Center for Human Immunology, Department of Medicine, Emory University School of Medicine, Room 1003 Woodruff Memorial Research Building, 101 Woodruff Circle, Atlanta, GA, USA

David L. Woodland
Trudeau Institute, Saranac Lake, NY 12983, USA

Deborah K. Dunn-Walters
Department of Immunobiology, 2nd Floor, Borough Wing Guy's, King's and St. Thomas School of Medicine, King's College London, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK

Delphine Sauce
Cellular Immunology laboratory, INSERM U543, Avenir Group, Hopital Pitie-Salpetriere, Université Pierre et Marie Curie-Paris, 91 Bd de l'Hopital, 75013 Paris, France

Dennis Taub
Clinical Immunology Section, Laboratory of Immunology, National Institute on Aging, National Institutes of Health, Baltimore MD 21224, USA

Domenico Lio
Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Donna M. Murasko
Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104, USA

Douglas E. Faunce
Department of Surgery and The Burn and Shock Trauma Institute; Department of Microbiology and Immunology; Loyola Aging and Immunology Program, Loyola University Medical Center, Stritch School of Medicine, Maywood, IL, USA

Elisa Cevenini
CIG-Interdepartmental Center “L. Galvani”, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy

Elisa Nemes
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Elissaveta Naumova
Central Laboratory of Clinical Immunology, University Hospital “Alexandrovska”. 1. G. Sofiisky str., 1431 Sofia, Bulgaria

Elizabeth J. Kovacs
The Burn and Shock Trauma Institute and the Immunology and Aging Program; Department of Surgery; Stritch School of Medicine, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA

Elizabeth M. Gardner
Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104, USA; Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, USA

Elke Richartz-Salzburger
Department of Psychiatry and Psychotherapy, University of Tübingen, Osiander-strasse 24, DE-72076 Tübingen, Germany

Emilie Combet
Section of Medicine and Therapeutics, Western Infirmary, University of Glasgow, Scotland

Enrico Lugli
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Eric J. Yager
Trudeau Institute, Saranac Lake, NY 12983, USA

Eric T. Clambey
Integrated Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Howard Hughes Medical Institute, National Jewish Research & Medical Center, University of Colorado Health Sciences Center, Denver, CO 80206, USA

Erika Roat
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Erminia Mariani
Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla-Putti, IOR, Via di Barbiano 1/10, 40136, Bologna, Italy

Esther Peralbo
Department of Immunology, Reina Sofia University Hospital, University of Córdoba, Spain

Eugenio Mocchegiani
Immunology Ctr., Section Nutrigenomic and Immunosenescence, Res. Dept. INRCA, Ancona, Italy

Ewa Bryl
Department of Pathophysiology, Medical University of Gdańsk, Poland

Ewa Sikora
Molecular Bases of Aging Laboratory, Nencki Institutew of Experimental Biology, Polish Academy of Sciences, Pasteura 3, Warsaw Poland

Flávia Ribeiro
Ageing and Tumour Immunology Group, University of Tübingen, Sektion Transplantionsimmunologie / Immunhämatologie, Waldhörnle Strasse 22, D- 72072 Tübingen, Germany

Florinda Listì
Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Frances T. Hakim
Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, MD, USA

Gary Van Zant
Department of Internal Medicine, University of Kentucky, Lexington, Kentucky, USA

Georg Wick
Division of Experimental Pathophysiology and Immunology, Laboratory of Autoimmunity, Biocenter, Innsbruck Medical University, Fritz-Pregl-Strasse 3, A-6020-Innsbruck, Austria

George C. Wang
Division of Geriatric Medicine and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, 5505 Hopkins Bayview Circle, John R. Burton Pavilion, Baltimore, MD 21224, USA

Gilles Dupuis
Clinical research Center, Department of Biochemistry, Immunology Graduate Programme, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada

Giuseppina Candore
Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Giuseppina Colonna-Romano
Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Graham Pawelec
Center for Medical Research (ZMF); Tübingen Ageing and Tumour Immunology Group, Center for Medical Research, University of Tübingen Medical School, Waldhörnlestr. 22, D-72072 Tübingen, Germany

Hartmut Geiger
Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

Haruyoshi Yamaza
Department of Investigative Pathology, Unit of Basic Medical Science, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Hideto Tamura
Division of Hematology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Hui-Chen Hsu
Department of Medicine, 1825 University Blvd, SHELB 310; The University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

I. Maeve Rea
Department of Geriatric Medicine, Queens University of Belfast, Northern, Ireland

Ian C. Brett
State University of New York, Stony Brook School of Medicine, Health Sciences Center, L4, Stony Brook, NY 11794, USA

Inmaculada Gayoso
Department of Immunology, Reina Sofia University Hospital, University of Córdoba, Spain

Isao Shimokawa
Department of Investigative Pathology, Unit of Basic Medical Science, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Jacek M. Witkowski
Department of Pathophysiology, Medical University of Gdansk, Gdansk, Poland

Jacob E. Kohlmeier
Trudeau Institute, Saranac Lake, NY 12983, USA

Jan Strindhall
Department of Natural Science and Biomedicine, School of Health Sciences, Jönköping University, Box 1026, 551 11 Jönköping, Sweden

Janet E. McElhaney
Geriatrics Research, University of British Columbia, Vancouver, Canada, and Center for Immunotherapy of Cancer and Infectious Diseases, University of Connecticut School of Medicine, Farmington, CT

Janet M. Lord
MRC Centre for Immune Regulation, Division of Immunity and Infection, Birmingham University Medical School, Birmingham B15 2TT, UK

Janko Nikolich-Zugich
Vaccine and Gene Therapy Institute, Department of Molecular Microbiology and Immunology and the Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006, USA

Jarrod Dudakov
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Javier G. Casado
Immunology Unit, Department of Physiology, University of Extremadura, Cáceres, Spain

Jean L. Scholz
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 36th and Hamilton Walk, Philadelphia, PA 19104-6082, USA

Jeff Leips
Department of Biological Sciences, 1000 Hilltop Circle, University of Maryland Baltimore County, Baltimore, MD, 21250, USA

Jens M. Nygren
Stem Cell Aging, Deptartment of Experimental Medical Science, BMC I13, Lund University, 221 84 Lund, Sweden

Jeremy Walston
Division of Geriatric Medicine and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, 5505 Hopkins Bayview Circle, John R. Burton Pavilion, Baltimore, MD 21224, USA

Jessica L. Palmer
Department of Surgery and The Burn and Shock Trauma Institute, Loyola University Medical Center, Stritch School of Medicine, Maywood, IL, USA

Jessica Reiseger
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Jian Chen
Department of Medicine, 1825 University Blvd, SHELB 310; The University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

John D. Mountz
Department of Medicine; Birmingham VA Medical Center, 1825 University Blvd, SHELB 310; The University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

John W. Kappler
Integrated Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Howard Hughes Medical Institute, National Jewish Research & Medical Center, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Departments of Medicine, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80206, USA

Jörg J. Goronzy
Kathleen B. and Mason I. Lowance Center for Human Immunology, Department of Medicine, Emory University School of Medicine, Room 1003 Woodruff Memorial Research Building, 101 Woodruff Circle, Atlanta, GA, USA

Jorge Lloberas
Institute for Research in Biomedicine-University of Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain

Joseph Lustgarten
Cancer Center Scottsdale, Mayo Clinic Arizona, 13400 East Shea Boulevard Scottsdale, AZ 85259, USA

Jürgen Kempf
Center for Medical Research (ZMF), University of Tübingen Medical School, Waldhörnlestr. 22, D-72072 Tübingen, Germany

Jyoti Misra Sen
Laboratory of Immunology, Clinical Immunology Section, National Institute on Aging, Intramural Research Program, National Institutes of Health, Baltimore, MD, USA

Kate L. Gibson
Department of Immunobiology, 2nd Floor, Borough Wing Guy's, King's and St. Thomas School of Medicine, King's College London, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK

Katerina Vlahos
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Katsuiku Hirokawa
Institute for Health and Life Sciences; Department of Comprehensive Pathology, Tokyo Medical & Dental University; Nakanosogo Hospital, Ascent Myogadani, Kohinata, Bunkyo-ku, Tokyo 112-0006, Japan

Kelly M. Hinkle
Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, Florida, USA

Kenneth H. Ely
Trudeau Institute, Saranac Lake, NY 12983, USA

Kiyoyuki Ogata
Division of Hematology, Department of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Ladislas Robert
Université paris 5. Laboratoire de Recherche Ophtalmologique., 1 place du Parvis Notre Dame, 75181 Paris cedex 04, France

Lara Gibellini
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Laura Bucci
Department of Experimental Pathology, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy

Laura Celani
CIG-Interdepartmental Center “L. Galvani”, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy

Leonarda Troiano
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Letizia Scola
Gruppo di Studio sull’Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Lia Ginaldi
Department of Internal Medicine and Public Health, University of L’Aquila, L’Aquila, Italy

Linda Bertoncelli
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Linda P. Fried
Division of Geriatric Medicine and Gerontology and Center on Aging and Health, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Lothar Rink
Institute for Immunology, RWTH Aachen University Hospital, Pauwelsstr. 30, 52074 Aachen, Germany

Lucia P. Mengoli
Department of Internal Medicine and Public Health, University of L'Aquila, L'Aquila, Italy

M. Luisa Pita
Department of Immunology, Reina Sofia University Hospital, University of Córdoba, Spain

Marcello Pinti
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Marcia A. Blackman
Trudeau Institute, Saranac Lake, NY 12983, USA

Marco Malavolta
Immunology Ctr., Section Nutrigenomic and Immunosenescence, Res. Dept. INRCA, Ancona, Italy

Maria Paola Grimaldi
Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Marilyn Armstrong
Department of Geriatric Medicine, Queens University Belfast, Belfast, Ireland, UK

Marius Wick
Department of Radiology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria

Masanobu Kitagawa
Department of Comprehensive Pathology, Aging and Developmental Sciences, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Masanori Utsuyama
Institute for Health and Life Sciences; Department of Comprehensive Pathology, Tokyo Medical & Dental University, Ascent Myogadani 4F, 4-4-22, Kohinata, Bunkyo-ku, Tokyo 112-0006, Japan

Massimo De Martinis
Department of Internal Medicine and Public Health, University of L'Aquila, L'Aquila, Italy

Mauro Provinciali
Laboratory of Tumour Immunology, INRCA Res. Dept., Via Birarelli 8, 60121 Ancona, Italy

Maximilian Zeyda
Department Internal Medicine III, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria

Michael P. Cancro
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 36th and Hamilton Walk, Philadelphia, PA 19104-6082, USA

Milena Ivanova
Central Laboratory of Clinical Immunology, University Hospital "Alexandrovska". 1. G. Sofiisky str., 1431 Sofia, Bulgaria

Milena Nasi
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Miriam Capri
Department of Experimental Pathology, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy; CIG-Interdepartmental Center "L. Galvani", University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy

Moisés E. Bauer
Faculdade de Biociências and Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga 6690, 2º andar. P.O. Box 1429. Porto Alegre, RS 90.610-000, Brazil

Nan-Ping Weng
Laboratory of Immunology, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA

Naozumi Ishimaru
Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3 Kuramotocho, Tokushima 770-8504, Japan

Natalie Seach
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Niklas Koehler
Department of Psychiatry and Psychotherapy, University of Tübingen, Osiander-strasse 24, DE-72076 Tübingen, Germany

Noweeda Mirza
Cancer Center Scottsdale, Mayo Clinic Arizona, 13400 East Shea Boulevard Scottsdale, AZ 85259, USA

Olivier Lesur
Clinical research Center, Graduate Immunology Program, Division of Pulmonology, Department of Medicine, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada

Owen A. Ross
Department of Geriatric Medicine, Queens University Belfast, Belfast, Irelan d, UK; Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, Florida, USA

Paul Moss
Institute for Cancer Studies, University of Birmingham, Vincent Drive, Birmingham B 15 2TT, UK

Pazit Beckerman
University Hospital Kerem, The Department of Medicine at the Hadassah Ein, Jerusalem, Isreal

Peter Uciechowski
Institute for Immunology, RWTH Aachen University Hospital, Pauwelsstr. 30, 52074 Aachen, Germany

Philippa Marrack
Integrated Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Howard Hughes Medical Institute, National Jewish Research & Medical Center, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Departments of Medicine, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80206, USA; Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, CO 80206, USA

Piotr Trzonkowski
Laboratory of Experimental Transplantology, Department of Histology and Immunology, Medical University of Gdańsk, Ul. Dębinki 1, 80-211 Gdańsk, Poland

Qing Yu
Laboratory of Immunology, Clinical Immunology Section, National Institute on Aging, Intramural Research Program, National Institutes of Health, Baltimore, MD, USA

Rafael Solana
Department of Immunology, Reina Sofia University Hospital, University of Córdoba, Spain

Raquel Tarazona
Immunology Unit, Department of Physiology, University of Extremadura, Cáceres, Spain

Richard Aspinall
Department of Immunology, Imperial College London, Faculty of Investigative Sciences, Chelsea and Westminster Campus, 369 Fulham Road, London, UK

Richard Boyd
Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Australia

Richard L. Riley
Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101, USA

Rita B. Effros
Department of Pathology & Laboratory Medicine, David Geffen School of Medicine at UCLA, 10833 Le Conte Avenue, Los Angeles, CA 90095-1732, USA

Rita Ostan
Department of Oncology and Experimental Pathology, University of Florence, Via Morgagni 50, Florence, Italy

Roberta Ferraresi
Chair of Immunology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, Modena, 41100, Italy

Sara Morgado
Immunology Unit, Department of Physiology, University of Extremadura, Cáceres, Spain

Sean X. Leng
Division of Geriatric Medicine and Gerontology and Center on Aging and Health, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Sergey G. Rudnev
Institute of Numerical Mathematics, Russian Academy of Sciences, Moscow, Russia

Simona Neri
Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla-Putti, IOR, Via di Barbiano 1/10, 40136, Bologna, Italy

Sirisha Karri
Division of Nutritional Sciences, The University of Texas, Austin, TX 78712, USA

Sonya Vasto
Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy

Stefano Salvioli
Department of Experimental Pathology, University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy; CIG-Interdepartmental Center "L. Galvani", University of Bologna, Via San Giacomo 12, I-40126 Bologna, Italy

Steven Castle
Geriatric Research Education and Clinical Center (GRECC) VA Greater Los Angeles Healthcare system, UCLA School of Medicine, 11301 Wilshire Boulvard, Los Angeles, CA, 90073, USA

Sudhanshu Agrawal
Division of Basic and Clinical Immunology, University of California, Irvine, CA 92697, USA

Sudhir Gupta
Division of Basic and Clinical Immunology, University of California, Irvine, CA 92697, USA

Susan E. McNerlan
Department of Geriatric Medicine, Queens University Belfast, Belfast, Ireland, UK

Takuya Chiba
Department of Investigative Pathology, Unit of Basic Medical Science, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Tamas Fulop
Centre de recherche sur le vieillissement; Research Center on Aging, Department of Medicine, Immunology Graduate Programme, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada

Tatiana A. Sannikova
Institute of Numerical Mathematics, Russian Academy of Sciences, Moscow, Russia

Thomas M. Stulnig
Department Internal Medicine III, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria

Toshimitsu Komatsu
Department of Investigative Pathology, Unit of Basic Medical Science, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Vanessa Nomellini
The Burn and Shock Trauma Institute and the Immunology and Aging Program; Stritch School of Medicine, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA

Victor Appay
Cellular Immunology laboratory, INSERM U543, Avenir Group, Hopital Pitie-Salpetriere, Université Pierre et Marie Curie-Paris, 91 Bd de l'Hopital, 75013 Paris, France

Wayne A. Mitchell
Department of Immunology, Imperial College London, Faculty of Investigative Sciences, Chelsea and Westminster Campus, 369 Fulham Road, London, UK

Wiebke Arlt
Division of Medical Sciences, University of Birmingham, Birmingham B15 2TT, UK

William J. Quinn III
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 36th and Hamilton Walk, Philadelphia, PA 19104-6082, USA

Yoshio Hayashi
Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3 Kuramotocho, Tokushima 770-8504, Japan

Yuko Kikuchi
Institute for Health and Life Sciences, Department of Comprehensive Pathology, Tokyo Medical & Dental University, Ascent Myogadani, Kohinata, Bunkyo-ku, Tokyo 112-0006, Japan

Part I
Methods and Models for Studying Immunosenescence

The Immune Risk Profile and Associated Parameters in Late Life: Lessons from the OCTO and NONA Longitudinal Studies

Anders Wikby, Jan Strindhall and Boo Johansson

Contents

Abstract: The OCTO Immune Longitudinal Study is a population-based study of ageing in a sample of 102 Swedish octogenarians with the aim to explore age changes of the immune system using a sample selected for good health. Data collection was performed in 1989, 1990, 1991 and 1997. An Immune Risk Profile (IRP) associated with increased mortality was characterized by high CD8+, low CD4+ T-cell counts and a poor T-cell proliferative response, inversion of the CD4/CD8 ratio and evidence of persistent cytomegalovirus infection was identified. The subsequent NONA Immune Longitudinal Study of 138 Swedish nonagenarians was performed in 1999, 2001, 2003, and 2005, not excluding individuals due to compromised health. The overall aim was to examine predictive factors for longevity and to further investigate in greater depth the immune risk profile identified in the OCTO Immune Study in the context of functional and disability parameters also examined

A. Wikby (✉) · J. Strindhall
Department of Natural Science and Biomedicine
School of Health Sciences
Jönköping University
Box 1026, 551 11 Jönköping, Sweden
Tel: +46-381-35101
Fax: + 46-381-36341
E-mail: anders.wikby@hhj.hj.se

B. Johansson
Institute of Gerontology, School of Health Sciences
Jönköping University, Box 1026, 551 11 Jönköping
Sweden, and Department of Psychology, Göteborg University
Box 500, 405 30 Göteborg, Sweden

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in the NONA. The immune panel included the analysis of T-cell subsets, inflammatory markers, virus serology, cytokines, TCR clonotype mapping, and functional and phenotypic analysis of virus specific CD8+ cells by HLA/peptide multimers, in collaborations between participants of the EU funded T-CIA project.

The present chapter report findings from the longitudinal studies of Swedish octo-nonagenarians with focus on IRP and its associations with persistent virus infection, CD8+ T-cell differentiation, cytokines, cognitive functioning, inflammatory activity, virus specific CD8+ cells, CD8+ T-cell clonal expansions and longevity. It also reports on low grade inflammation processes of importance in predicting longevity in the very late life.

Keywords: Immune risk profiles • Immunosenescence • Longitudinal studies • T-cells

1 Introduction

The very old constitute the fastest growing age segment in developed countries. From a societal and population perspective, this demographic trend is also accompanied by an increase in the number of very old individuals with compromised health and significant requirements for service and health care. From a physiological perspective, the robustness of the immune system is particularly important in this age segment, considering the fact that the incidence of death due to infection diseases seems to continue to increase although mortality related to cardiovascular disease and cancer may level off in many populations (Vasto et al. 2007).

Immune studies of elderly populations, however, so far have mainly been conducted on individuals in their 60s and 70s. Few studies have focused on samples over 80 years and still fewer have employed longitudinal designs that allow studies of intra-individual change (Pawelec et al. 2005). In the Swedish OCTO and NONA Immune Longitudinal Studies (Wikby et al. 1994, 2002), we deliberately examined individuals in very late life because of the substantially elevated risk for compromised health, morbidity, and mortality. The overall aim was to provide better understanding of processes and mechanisms related to intra-individual change in various parts of the immune system regulation in very late life. An aim was also to identify presumptive predictors for subsequent mortality and clinical parameters related to the substantial morbidity/comorbidity observed in late life. From a clinical perspective detection of predictive markers may enable interventions that could assist in various improvements of quality of life for individuals in this rapidly growing age segment.

The OCTO Immune Longitudinal Study is a population-based study of ageing and the immune system in a sample of Swedish octogenarians (Wikby et al. 1994). It was started in 1989 in Jönköping, Sweden, as a collaboration between researchers at the Institute of Gerontology and the Department of Natural Science and Biomedicine, School of Health Sciences, Jönköping University, the Department of Micro-

biology, Hospital of Ryhov, Jönköping and the Department of Veterinary Science, Penn State University, USA and ended in 1997 when the vast majority participants were deceased. The subsequent NONA Immune Longitudinal Study of nonagenarians was initiated in 1999 to extend and refine findings from the OCTO Immune Longitudinal Study identifying an Immune Risk Profile (IRP) associated with an elevated mortality rate (Wikby et al. 2002). The NONA immune also became part of the EU supported programs *Immunology and Ageing in Europe, ImAginE,* (Pawelec, Caruso 2003) and *T cell immunity and ageing, T-CIA,* (Koch et al. 2005) creating collaborations between the NONA immune researchers and several European laboratories participating in these networks. The OCTO-NONA Immune Longitudinal Studies have investigated predictive factors for longevity with focus on immune risk profiles in a context of functional and disability health parameters of importance in late life. The present review summarizes some of the main findings and lessons learned from these studies.

2 Methodological Design and Sampling Considerations in Ageing Studies

2.1 Design Considerations

First we address the significant design and sampling considerations that directed our research. The two methods used in population-based studies of ageing are the cross-sectional and longitudinal designs (Wikby et al. 2003). The most common design is the cross-sectional, in which two or more age groups are compared at a single occasion. Age changes are typically inferred from the observed age differences in mean values. This design provides a procedure that is logistically easy and fast and less expensive than the longitudinal design. However, great caution is necessary in the interpretation of cross-sectional data since age differences may be confounded by the fact that birth-cohorts have been exposed to various environmental exposures and socio-cultural influences (Wikby et al. 2003; Pawelec 2006). Another confound that become more of an issue with age is that of selective mortality (Wikby et al. 2003). As a study population ages it becomes gradually more selected, since deaths do not occur at random. For example, if a high value in a variable is deleterious, death is likely to occur first in individuals with high values and last in individuals with low values. In a cross-sectional study an observed difference in mean values between age groups may be incorrectly interpreted as a real age change rather than as an effect of selective mortality. Many studies have characterized changes in the immune system with age, but a number of these have yielded conflicting results, partly due to the fact that the vast majority of these studies are cross-sectional (Wikby et al. 2003).

In a longitudinal design (Wikby et al. 2003) individuals are followed across time, usually with a number of years in between measurement occasions. This allows

the detection of intra-individual change and minimizes many of the confounding artefacts likely to emerge in the cross-sectional design. Although the longitudinal design represents the superior alternative for conducting ageing research, the use of this design has been very limited, particularly in studies of the immune system. The main reason is that such studies are expensive and require considerable effort, financial support, and commitment of personnel. In addition, longitudinal studies require careful coordination, standardised procedures, and control of studied panels to avoid dropouts. A main caution to note in the use of a longitudinal design is the involvement of a possible confounding between age and time of measurement effects. Time of measurement confounding includes numerous factors, such as the motivation and interest of the participating subjects, experimenter effects including changes in personnel and their motivation, and in the methods, techniques and essays used across time. Many of these problems can be compensated for by including a younger group for comparisons across measurement occasions. The immune system changes that occur across times of measurement will then be negligible in the healthy young people compared to the very old. Also, restricted time periods between the measurements and the use of identical methods will prevent time of measurement effects.

2.2 Sampling Considerations

Advancing age is typically accompanied by an increased prevalence of compromised health and diseases (Jeune 2002). This is one of the primary problems in the selection and definition of a sample in population-based studies of ageing. To overcome this problem, most studies have used various selection schemes to exclude individuals with underlying diseases from participation in studies of the immune system. The stringent *SENIEUR* Protocol (Ligthart et al. 1984) represents an example of a widespread application of a set of exclusion criteria used to select individuals in good health, to be able to distinguish between age changes caused by *primary ageing* and *secondary ageing*, i.e. by diseases. Noteworthy, the exclusion of *non-SENIEUR* individuals will, however, result in a study of less than 10% of a population among individuals aged over 80 years and older (Pawelec et al. 2001). Another way to diminish confounding between primary ageing effects and disease has been to employ exclusion criteria tailored to the experimental situation (Hallgren et al. 1988), i.e. in immune studies to exclude individuals that have immune related diseases or who use drugs that affect the immune system. Such a strategy was used in the OCTO Immune Longitudinal Study but will also generate a select sample. In our case, about 50% in a population aged over 80 years were excluded (Wikby et al. 1994).

A way to overcome some of the selection problems is to examine a population-based sample, combined with careful continuous evaluation of individual health parameters (Nilsson et al. 2003). This was the approach taken in the NONA Immune Longitudinal Study. The clinical variables needed for the evaluation of

individual health and morbidity are then of considerable value in the comparison of findings from the application of various protocols and in the categorization of individuals into subgroups according to their health status (Nilsson et al. 2003). Thus, the significance of a change in health status is included rather than excluded as an important consideration in these aging studies.

3 The OCTO and NONA Immune Longitudinal Studies

3.1 The OCTO Immune Study

The OCTO Immune Longitudinal Study was an integrated part of the OCTO Longitudinal Study of biobehavioral ageing, in Jönköping, Sweden. The municipality of Jönköping has 122 000 inhabitants and is situated in South-central part of Sweden. The aim of the OCTO immune was to explore age changes in the immune system in Swedish octogenarians relative to an array of medical, biobehavioral, and social variables (Wikby et al. 1994).

Census data was used to identify octogenarians living in Jönköping and born in 1897, 1899, 1901, and 1903. A non-proportional sample that composed of 100 persons in each of the birth-cohorts was recruited. From these 400 individuals, 324 were examined in the first wave in 1987/1988 of the OCTO study. The persons were then at the ages of 84, 86, 88, and 90 years old. At the second wave of the study, the OCTO Immune Longitudinal Study was initiated. Of the 324 examined at baseline of the OCTO, 96 were deceased before the start of the second wave of this study. Another 15 declined to participate, giving a total number of potential participants of 213 for the OCTO immune.

Exclusion criteria were set to diminish confound between ageing, disease, and medications and to secure reliable psychosocial self-reports. Potential candidates were included if they:

- Were noninstitutionalized
- Had normal cognition according to neuropsychological tests (Johansson et al. 1992)
- Were not on a drug regimen that may influence the immune system.

These exclusion criteria were similar to those of Hallgren et al. (1988). Of the potential 213 individuals, 110 met inclusion criteria. Of these, 102 individuals participated in the first wave. Sixty-nine individuals were available throughout the three waves in the longitudinal analysis and 23 participated in the longitudinal analysis over all four time-points, T1 (1989), T2 (1990), T3 (1991), and T4 (1997) (Table 1). Nonparticipation at the various measurement occasions was mainly due to mortality in the sample. Fourteen healthy middle-aged volunteers (39 years SD±5.8) of men and women working in the laboratories at Ryhov Hospital in Jönköping were included across the measurement occasions for comparative reasons.

Table 1 Characteristics of individuals included in the OCTO Immune Longitudinal Study

Occasion (Time)	Year	Number of individuals investigated	Age (years)	
			Mean	Range
1	1989	102	88	86–92
2	1990	83	89	87–93
3	1991	69	90	88–94
4	1997	23	95	94–100

The very old individuals were examined in their place of residence. Blood samples were drawn in the morning between 8:00 and 10:00 (a.m.). The following immune system parameters were investigated:

- Complete blood cell count
- Differential WBC count
- Antibody defined T and B cell surface molecules using three colour flow cytometry
- Proliferative response of PBMC using a mitogen stimulation assay with ConA in cell culture
- Interleukin 2 production
- Cytomegalovirus (CMV) and Herpes simplex serology.

3.2 The NONA Immune Study

Findings from the OCTO Immune Longitudinal Study constituted the background for the subsequent ongoing NONA Immune Longitudinal Study of nonagenarian individuals also living in the municipality of Jönköping (Wikby et al. 2002). The NONA immune is an integrated part of the NONA Longitudinal Study initiated to examine the disablement process in late life. The overall aim in the NONA immune is to examine predictive factors for longevity in the very old and to further investigate in greater depth the immune risk profile identified in the OCTO immune. The aim is also to consider immune data in the context of functional and disability parameters examined in the overall NONA. The overall study includes measurements of the following functional and disability domains:

- Physical and mental health
- Cognitive functioning
- Personal control/coping
- Social networks
- Provision of service
- Care and everyday functioning capacity.

The NONA immune examines a population-based random sample without excluding individuals due to compromised health, but to include a continuous evaluation of various individual health parameters (Nilsson et al. 2003). Individuals

were drawn from the population (census) register of Jönköping. A nonproportional random sampling procedure was employed, including all individuals permanently residing in the municipality, with the goal to have individuals aged 86, 90, and 94 years old. The sampling frame was defined on the available census information in September 1999. As the number of available subjects in the oldest birth cohort was limited, a few subjects were also included from the birth cohorts of 1904 and 1906. Blood samples for the immune system analysis were drawn in 138 individuals, of whom 42 belonged to the oldest birth cohort, 47 were 90-years, and 49 86-years old. Data collections were made using two-year inter-occasion intervals in 1999, 2001, 2003, and 2005.

The mean age of the sample at baseline was 89.8 years with a total proportion of women of 70%. While about 60% of them lived in an ordinary housing, 40% resided in a sheltered housing or in institution. A comparison between individuals who participated in the in-person testing part of the NONA study (n=157), and those who accepted that blood was drawn (n=138), indicated no significant differences for demographics or overall ratings of physical and mental health. In the second wave, 61% of individuals participated, at the third 40%, and at the fourth only 22%. Nonparticipation at the various measurement occasions was mainly due to mortality. A younger group of 22 healthy middle-aged men and women working at the Ryhov Hospital in Jönköping participated (mean age 44.7, SD=8.9 at baseline) across measurement occasions for the sake of comparison. Characteristics of the individuals participating in the NONA Immune Longitudinal Study are summarised in Table 2.

Health was defined based on medical records and from clinical chemistry data, supplemented with information gathered in a health interview that focused on diagnosed illness, current symptoms, and use of medications (Nilsson et al. 2003). The neuropsychological battery used to identify cognitive impairment included the Mini-Mental State Examination (MMSE) and the Memory-In-Reality (MIR) test (Folstein et al. 1975, Johansson 1988/1989). MMSE is a screening device used in epidemiological studies to identify cognitive impairment. The MIR test comprises of a naming condition for 10 common real-life objects, followed by showing a three-dimensional model of an apartment. The participants are then asked to place the objects in the different rooms according to personal preferences. Following a distraction, a recall test is administered, followed by a recognition task for items not recalled. In the NONA Immune Longitudinal Study we used the following three

Table 2 Characteristics of the subjects participating in the NONA Immune Longitudinal Study

Occasion (year)	No. of subjects investigated	Proportion of women (%)	Age (years)	
			Mean	Range
1999	138	70	89.8	86–95
2001	84	69	91.6	88–97
2003	55	69	93.2	90–99
2005	31	81	94.7	92–101

cognitive status categories: 1) cognitive intact, 2) mild cognitive dysfunction or questionable cases (MCD, evidence of compromised memory/cognition, not fully meeting DMC-IV criteria for dementia, APA, 1994), and dementia (according to DMS-IV criteria, APA 1994). The two latter diagnostic categories were pooled under the category of "cognitive impairment" and compared with those rated as cognitively intact.

Subjects were examined in their place of residence by trained Registered Nurses with extensive experience of working with the elderly. The tests and interviews took about 3 hours, including breaks, for individuals who were able to participate in all parts. The blood samples were drawn in the morning between 09:00 and 10:00. The following immune and clinical components are studied in the NONA Immune Longitudinal Study:

- Complete blood cell count
- Differential WBC count
- Proteins, albumin, transthyretin, C-Reactive Protein, orosomucoid, haptoglobulin,
- IgG, IgM, IgA, urea, cystatinC, creatinine as indicators of malnutrition, inflammation or kidney disease
- Antibody defined T-cell surface molecules of T, NKT, NK cell populations, using three colour flow cytometry
- Secretion of cytokines, IL-2, IL-6, IL-10, interferon-gamma
- CMV, EBV and Herpes simplex serology
- MHC/peptide tetramers to analyze the number of CMV and EBV specific CD8+ cells
- TCR clonotype mapping with Denaturing Gradient Gel Electrophoresis (DGGE), including RNA extraction, cDNA synthesis and amplification by use of a primer panel amplifying the 24 BV region families covering a majority of TCR's. The resulting DNA fragments are separated by DGGE and expanded clones are identified as distinct bands on a gel (thorStraten et al. 1998).

4 Results and Discussion

4.1 The OCTO Immune Study

In the OCTO Immune Longitudinal Study we were able to identify an immune risk profile by multiple comparisons of individuals grouped by homogeneity of certain combinations of adaptive immune system parameters (Ferguson et al. 1995). These cluster analysis use profile similarities to group individuals when the number and nature of the groups are not known in advance, ideal in the exploration of complex systems like the immune system. The analysis was employed to determine groups based on immune functioning and T-cell subpopulations using the mitogen response to Concanavalin A, and the percentages of CD3, CD4, CD8, and CD19 positive cells. The groups identified by cluster analysis were then compared with respect

to their impact on survival-non-survival by chi-square analysis. This analysis of immune data at baseline revealed an Immune Risk Profile (IRP) predictive of subsequent 2-year mortality (Ferguson et al. 1995). An IRP cluster, designated cluster 1, was characterized by immune parameters that consisted of high levels of CD8+ T-cells, low levels of CD4+ and CD19+ T-cells, and poor proliferative mitogen response to ConA (Table 3). No such association could be found using common methods for univariate analysis.

The result demonstrated that additional individuals developed the IRP by increases in the CD8+ cells as well as decreases in the CD4+ cells and CD4/CD8 ratio between baseline and a 2-year follow-up (Wikby et al. 1998). At that time the IRP individuals again were found to have increased subsequent 2-year mortality. Interestingly, we found that the IRP could be defined by using only the inverted CD4/CD8 ratio, since this sole marker was strongly associated with the IRP defined by the cluster of parameters (Wikby et al. 1998).

The results also showed that 31% individuals out of the 102 participating either had at baseline (16%) or developed (15%) an Immune Risk Profile during the 8-year longitudinal period of the study (Olsson et al. 2000). Noteworthy, individuals who belonged to the IRP category at baseline or moved into that category over the 8 years never moved out from this elevated mortality risk group (Olsson et al. 2000).

Although the significance for changes leading to a skewed CD4/CD8 ratio in the IRP was not well understood at the time of our initial exploration, the relationship observed between a reduced functional immune response and mortality had indeed been described in several previous studies. It was reported in humans that with age the lack of a response to three mitogens: the T-cell mitogens concanavalin A, phytohemagglutin, and the T-dependent B-cell mitogen, pokeweed, were associated with increased mortality (Murasko et al. 1987). In another study of individuals older than 80 years of age, it was found that anergic aged individuals had a 2-year mortality rate of 80% compared to 35% in those who were nonanergic (Roberts-Thomson et al. 1974). A third study examined the relation between anergy and all cause mortality in healthy individuals above 60 years of age (Wayne et al. 1990). The study showed that anergy, defined as a decreased delayed type hypersensitivity (DTH) response in a skin test to four common recall antigens, was associated with nonsurvival.

Since our study at baseline did not analyse subsets of CD4 and CD8 T-cells on the basis of other phenotypic markers, the changes in the CD4/CD8 balance in IRP individuals was not well characterized. In 2000 various subsets of CD4 and

Table 3 Statistical description of variables used in the formation of a three cluster solution

Cluster (n)	Mitogen response/DPM	CD3+/%	CD4+/%	CD8+/%	CD19+/%
1 (14)[a]	11077 (8413)[b]	62.6 (14.8)	30.8 (4.3)	43.3 (8.9)	5.5 (2.6)
2 (36)	16915 (11491)	75.6 (7.6)	47.9 (12.1)	26.5 (5.9)	8.4 (4.1)
3 (39)	29681 (14427)	54.5 (12.3)	42.4 (9.8)	20.5 (6.9)	12.5 (7.1)

[a] IRP cluster predicting non-survival
[b] Mean (SD)

CD8 were therefore included in the study (Olsson et al. 2000). The results indicated immune system changes that suggested a loss of T-cell homeostasis, as reflected by a substantial increase in the number of CD8 cells with parallel decrease in the number of CD4 cells in individuals with an inverted CD4/CD8 ratio. The changes were apparent in a number of T-cell subsets, with significant increases in the levels of CD8+CD28- cells, in particular, demonstrating that differentiated effector/memory CD8+ cells are disproportionately represented in this cell population. These cells has been shown by others have shortened telomers, suggesting an extensive history of replication (Effros 2007). Initially it was surprisingly found that these homeostatic T-cell changes associated with an inverted CD4/CD8 ratio was associated with persistent CMV infection, prevalent (90%) in the very old (Olsson et al. 2000). Importantly, our studies showed no evidence of a relationship of these T-cell changes and other viruses, Herpes simplex and Epstein Barr viruses, indicating an unique impact of CMV on the immune system. This result was unexpected since the carriage of CMV had long been considered to be quite harmless to individuals with a functional immune system. The finding thus suggested that the changes in the T-cell balance among IRP subjects at least partly is produced by the generation of CD8+ effector/memory cells against persistent CMV infection and subsequent homeostatic decreases in the CD4+ and CD4/CD8 ratio. This conclusion was supported by tetramer technology demonstrating significant expansions of CD8+ T-cells specific for the CMV_{NLV} peptide in HLA-A2 individuals to be associated with both age and the IRP (Ouyang et al. 2004).

4.2 The NONA Immune Study

Results from the OCTO Immune Longitudinal Study provided the basis for the subsequent Swedish NONA Immune Longitudinal Study (Wikby et al. 2002) and potentials to further advance and refine our knowledge about various predictive factors for longevity but still with special focus on the Immune Risk Profiles (IRP's). The NONA sample provided a broader set of functional and disability parameters, including morbidity, cognitive impairment and chronic viral infection, to be examined in relation to longitudinal changes in inflammatory parameters, the CD8+ T-cell phenotype and differentiation, and CD8+ T-cell clonal expansion.

4.2.1 Immune Parameters and Morbidity

Studies of the immune system in very old individuals are most commonly performed on highly selected samples by the use of selection protocols excluding individuals with conditions that influence the immune system (Nilsson et al. 2003). Among a great variety of protocols the SENIEUR protocol represent the most commonly used and accepted with a comprehensive set of health and laboratory criteria for sample selection aiming at the distinguishing between ageing per se and

those associated with morbidity (Lighthart et al. 1984). Another selection protocol used in the studies of ageing and the immune system, used in the Swedish OCTO Immune Longitudinal Study, is that proposed by Hallgren et al (1988). This protocol excludes individuals with diseases and other conditions known to affect specifically the immune system to tailor the study to its particular purpose. In the NONA Immune Longitudinal Study a slightly modified SENIEUR and Hallgren protocol were used to characterize the sample according to health status (Nilsson et al. 2003). This permitted us to distinguish subgroups of very healthy, moderately healthy and frail individuals for various immune system parameter comparisons.

The modified SENIEUR protocol excluded 90.6% of the NONA immune sample at baseline, indicating that only 9.4% were rated as *very healthy*. The use of the original protocol, suggesting additional laboratory analysis for exclusion, would probably have excluded even more individuals, demonstrating the need for using less stringent criteria in studies of the immune system in later life to avoid studies of only highly selected, nonrepresentative samples. Thirty-eight (27.5%) participants, selected from those being not very healthy and defined as *moderately healthy*, met the criteria used in the previous OCTO Immune Longitudinal Study of not residing in an institution, not being demented, and not using medication known to affect the immune system. The remaining sample (63%) comprised *frail* individuals not meeting the above health criteria (Nilsson et al. 2003).

Applying the five most common exclusion criteria, cardiac insufficiency, medication, laboratory data, urea and malignancy, the modified SENIEUR protocol excluded 87% of the original sample (Nilsson et al. 2003). When the OCTO Immune protocol was applied, medications was found to be the most common criterion, excluding 43%, institutionalisation the second, excluding 39%, and cognitive dysfunction the third, excluding 14%. Among various diseases conditions cardiac insufficiency (51%), malignancy (15%), dementia (14%), chronic obstructive pulmonary disease (12%), diabetes mellitus (11%), rheumatoid arthritis (9%), hypothyroidism (6%) and pernicious anaemia (6%) constituted the eight most prevalent diagnoses. These figures demonstrate the considerable prevalence of morbidity and comorbidity in a representative sample of very old individuals (Nilsson et al. 2003).

A comparison of the number of T-cells across the subgroups of very healthy, moderately healthy and frail indicated no group differences for subsets characteristic of the immune risk profile, previously identified in octogenarians (Nilsson et al. 2003). Interestingly, the IRP might thus serve as a significant biomarker of ageing, independent of overall health status. This is further confirmed by results demonstrating that clusters of immune markers can predict longevity in noninbred mice independently of health conditions (Miller 2001).

4.2.2 Immune Risk Profile, Cognitive Impairment and Mortality

Prevalence and incidence of cognitive impairment and dementia become substantial in very old people. Studies have shown that compromised cognition is significantly related to proximity of death by a twofold increased mortality risk among demented

octogenarians and nonagenarians (Johansson and Zarit 1997; Wilson et al. 2003). There is also considerable evidence suggesting interactions between the nervous and innate immune systems, in which cytokines have a central role as communicators (Wilson et al. 2003). Studies have suggested that higher levels of interleukin 6 (IL-6) are significantly associated with poorer cognitive function and predict future cognitive decline among the elderly (Marsland et al. 2006). In pathological conditions such as ischemia and Alzheimer's disease, microglia cells in the brain seem to respond to injury by producing increased levels of particularly the proinflammatory cytokines interleukin 1 (IL-1) and the multifunctional IL-6 (Tarkowski 2002).

Analysis of mortality in the very old NONA immune individuals (n=138) confirmed our previous findings in the OCTO Immune Longitudinal Study of an approximately twofold mortality rate in the 22 (16%) individuals with an IRP, i.e. showing a significantly higher relative 4-year mortality (77%) than those who were non-IRP individuals (43%), a finding suggesting that the IRP concept could be generalized to the more broadly defined NONA sample (Wikby et al. 2005). The findings was also in line with the Healthy Ageing Study in the Nottingham/Cambridge area in the UK in which it was found that an inverted CD4/CD8 ratio is predictive of nonsurvival in older adults (Huppert et al. 2003).

Our results also supported previous findings in samples of octogenarians and nonagenarians of a twofold elevated mortality risk in individuals with cognitive impairment (Wikby et al. 2005). Among the NONA Immune individuals (n=138), those who were categorized as cognitively impaired (29%) also showed a significantly higher 4-year mortality (75%) compared with cognitively intact individuals (39%). Moreover, the results showed that the two conditions of IRP and cognitive impairment independently predicted survival also when age, sex and various kinds of prevalent diseases and comorbidity were controlled for (Wikby et al. 2005). This provided further support for the previous findings that IRP constitute a major predictor of nonsurvival in very late life independently of morbidity. Only 9% of the NONA Immune individuals conformed to the SENIEUR criteria for optimal health (Nilsson et al. 2003).

4.2.3 Allostatic Load

The concept of allostatic load was proposed by McEwen and Stellar as a measure of dysfunctions across multiple physiological systems, suggesting that the cumulative dysfunctions may have more than an additive impact on overall health and survival (McEwen and Stellar 1993). Allostatic load derives from the concept of allostasis which in turn is derived from homeostasis (McEwen 2003). Allostasis, however, focus more specifically on the challenges upon the specific regulatory nervous, immune and endocrine systems in order to adapt to maintain balance though changes in various psychosocial or physical situations, like stress, in life (Karlamangla et al. 2002). Although such processes may be adaptive in the short term, they are likely to be damaging when becoming excessive in duration, frequency and magnitude (McEwen 2003). This line of thinking correspond to the growing interest to identify

more comprehensive measures that incorporates multiple risk factors that may predict subsequent health and survival (Karlamangla et al. 2006).

In the NONA Immune Longitudinal Study we identified a small sample (n=8) with both IRP and compromised cognitive status at baseline (Wikby et al. 2005). A Kaplan-Meier survival analysis revealed that these individuals showed a significantly higher annual mortality rate (42%/year) compared with those with one of the conditions (15%/year) as well as with those having none (8.5%/year), corresponding to relative mortality rates of 5:2:2:1 (Fig. 1). These observed mortality effects indicates immune and central nervous system interactions, and were integrated into the general framework of allostatic load, since survival data suggested that the cumulative dysfunctions across the nervous and immune systems had more than an additive impact on survival (Wikby et al. 2005).

The allostatic load in IRP individuals with cognitive impairment was associated with changes in the levels of the cytokines IL-2 and IL-6 (Wikby et al. 2005). Cytokines in general are considered to have a central role in the mediations of allostasis by communications between the nervous, immune and endocrine systems (McEwen 2003). A suppression of the T-cellular function in IRP individuals is supported by our finding of poorer IL-2 responsiveness in those individuals compared with non-IRP's (Wikby et al. 2005). A further decline of this responsiveness in IRP individuals with cognitive impairment support the existence of an interaction between the nervous and peripheral immune system dysfunctions with a further down-regulation of the T-cellular response in these persons. Excessive increases in the plasma levels of the proinflammatory cytokine IL-6 did also represent changes characteristic of an allostatic load in the individuals and might have contributed to the T-cellular suppression by acting as an immunosuppressant via the hypothalamic-pituitary-adrenal axis (Wikby et al. 2005).

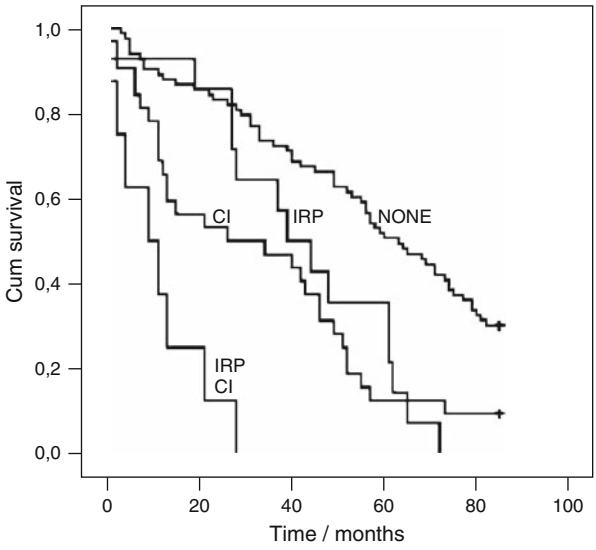

Fig. 1 Kaplan Meier survival curves for NONA individuals in subgroups created by IRP status combined with cognitive status. The subgroups were: "IRP, CI" (IRP, cognitively impaired); "CI" (cognitively impaired, non-IRP); "IRP" (IRP, cognitively intact); "NONE" (non-IRP, cognitively intact). Test for equality of survival distribution for the subgroups showed, p<0.001

4.2.4 IRP, T-cell Differentiation and Persistent Viral Infection

Baseline results also confirmed findings from the OCTO immune study that showed an association between the IRP and the prevalence of persistent CMV infection (Wikby et al. 2002). As for the OCTO immune study, the NONA study demonstrated a CD3+CD8+CD28-phenotype as markedly expanded for IRP and CMV-positive individuals. This led us to examine the T-cell differentiation in more detail, using the CD45RA+, CCR7+, CD27+ and CD28+ markers in a sequential model, suggesting a positive expression for naive cells, gradual losses of the markers in the various memory stages and negative expression for lately differentiated effector/memory cells (Appay et al. 2002, Akbar, Fletcher 2005). A final differentiation step occurs by reversion of CD45RO+ to CD45RA+ to obtain CD27-CD28-CCR7-CD45RA+ terminally differentiated cells of effector type (Wallace et al. 2004).

Our results suggested major decreases in the number of naive cells in the very old, changes that were even more pronounced in IRP individuals (Fig. 2). The results also showed significant increases in the number of CD8+CD27-CD28-CCR7-perforin+ effector/memory and effector cells in IRP individuals (Fig. 2) and since a majority of these cells also were CD45RA+, data confirmed that the IRP is strongly associated with increases in the number of terminally differentiated effector cells. Recent evidence suggests that increased proportions of terminally differentiated CD8+ cells possess characteristics of replicative senescence, including telomere shortening and apoptosis-resistance (Effros 2007). The inclusion of high proportions of senescent T-cells in the IRP may for the first time provide clinical confirmation of the Hayflick Limit theory of human ageing (Effros 2004). The clinical relevance for the prevalence of large amounts of senescent CD8+ T-cells has also been demonstrated by three independent studies performed on different elderly populations (Goronzy et al. 2001; Saurwein-Teissl et al. 2002; Trzonkowski et al. 2003. These studies showed consistently that a diminished antibody response to influenza vaccination is significantly associated with having high proportions of a population of CD8+ cells that lack expression of the costimulatory molecule CD28.

Evidence for a major impact of CMV in generating terminally differentiated CD8+ cells was demonstrated in the OCTO subjects by tetramer technology and was also confirmed in the NONA Immune Study (Reker-Hadrup et al. 2006). We found CMV_{NLV} specific expansions, mainly composed of terminally differentiated cells, in the range 1–20% of total CD8+ cells, similarly to findings in the OCTO Immune Study. Increases in the CMV_{NLV} percentages were associated with decreases in the IFN-γ responsiveness, suggesting that the accumulation of CMV-specific T-cells is a result of compensatory mechanisms to control CMV to balance the compromised functionality that occur with increasing age (Reker-Hadrup et al. 2006). Recent findings have indicated a failure in this control by indicating that the aged immune system is unable to control CMV and EBV, supporting the view that the expansion of virus-specific CD8+ T-cells might be due to increased herpes virus reactivation and replication (Stowe et al. 2007).

The NONA immune results also support the suggestion that besides CMV infection, persistent EBV infection plays a role as bystander associated with the

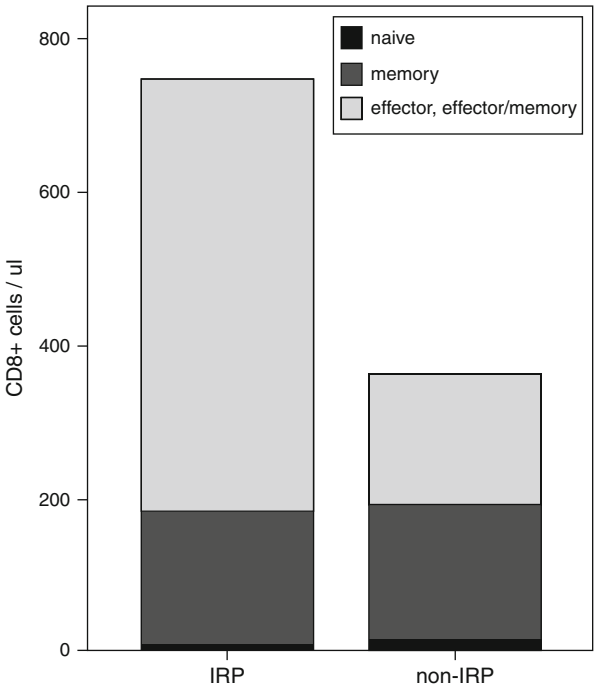

Fig. 2 Mean number of CD8+ T-cells (per µl) and subsets of CD8+ differentiation in IRP and non-IRP NONA individuals. Naïve cells were identified as CD8+CD45RA+CCR7+ cells, memory cells as CD8+CD28+CD27+ CD45RA-, CD8+CD28+ CD27-, CD8+CD28-CD27+ cells, and effector, effector/memory cells as CD8+CD28-CD27-CCR7- cells. The number of effector, effector/memory cells was significantly ($p<0.001$) higher in IRP individuals compared with non-IRP individuals

IRP (Wikby et al. 2005). IRP individuals were in all cases double sero-positive, suggesting that chronic viral load in the very old might contribute to the development of an IRP. Increased numbers of lately differentiated CD8+ cells, characteristic of the IRP, was also found particularly in double sero-positive individuals, to a less but significant extent in those being infected with CMV only, and to a low extent in individuals only infected with EBV (Wikby et al. 2005). In line with this we found significant expansions of EBV_{GLC} specific CD8+ cells; although their frequency was tenfold lower than for the CMV-specific cells (Ouyang et al. 2003).

4.2.5 TCR Clonotype Mapping

Clonal expansions have been detected in healthy old individuals and accumulating evidences suggest that that these expansions are associated with chronic antigen stress induced by persistent viral infections (Khan et al. 2002). We analysed the CD8+ T-cell clonal composition in NONA immune (n=39) and middle-aged (n=9) individuals using TCR clonotype mapping (Reker-Hadrup et al. 2006). The method combines RT-PCR and denaturing gel electrophoresis (DGGE) for rapid detection and characterization of T-cell clonal expansions by use of specific primers covering a vast majority of TCRBV 1–24 variable regions (thor Straten et al. 1998). With a polyclonal T-cell population a nondistinct smear in the denaturing

gradient gel is seen while, in contrast, a population of clonally expanded TCR is seen as a distinct band. The clonal expansion were quantified by staining with anti-TCR-BV mAbs showing that for an individual CMV_{NLV} specific clone to be detected as expanded, the clone exceeds at least 1% of the CD8+ repertoire Reker-Hadrup et al. 2006).

The mean number of expanded clones was significantly higher in nonagenarians compared with the middle-aged (Fig. 3), suggesting a considerable impact of CD8+ clonal expansions in the very old (Reker-Hadrup et al. 2006). Importantly, these clonal expansions were also found to be stable across a two-year period of time. The results also showed a very strong association between the number of expansions and persistent CMV infection (Fig. 3), suggesting that a vast majority of CD8+ clonal expansions in the elderly are derived from CMV. Direct evidence for this was also demonstrated, since the sorting of CMV_{NLV} specific cells and subsequent TCR clonal mapping revealed that this specific T-cell population was oligoclonal with a mean number of six CMV related clone types (Reker-Hadrup et al. 2006). These results are comparable with findings showing that when a broad range of CMV epitopes was studied by tetramer technology, the aggregated percentages of the specific cells were more than 10% and as high as 50% of the total number of CD8+ cells (Moss and Khan 2004). Such substantial accumulations of CMV specific cells in a limited number of clones may reduce the available space for T-cells with other specificity, which may be lost through competition and result in a reduced clonal diversity and immune protection capability, particularly relevant for IRP's (Akbar and Fletcher 2005). A demonstration that clonal expansions of specific T-cells can compromise the response to other antigens by a mechanism through competition was given in mice (Messaoudi et al. 2004). The

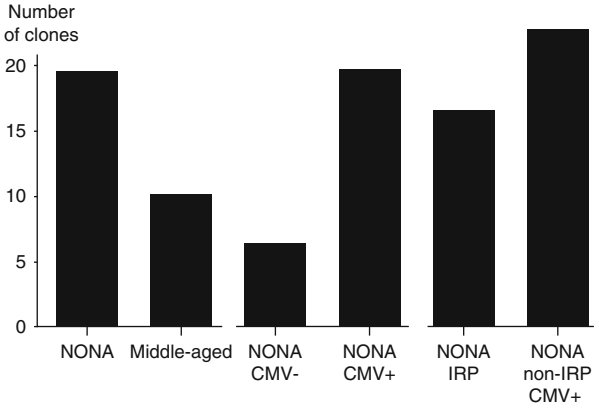

Fig. 3 The mean number of clonal expansions in the CD8+ repertoire determined by DGGE in subgroups of individuals. NONA individuals showed significantly ($p<0.01$) higher mean number (19.4, $n=39$) compared with middle-aged (10.1, $n=9$). CMV positive NONA individuals showed significantly ($p<0.001$) higher mean number (22.6, $n=31$) compared with CMV negative NONA individuals (7.4, $n=8$). CMV-positive NONA IRP individuals showed significantly ($p<0.05$) lower mean number (15.0, $n=8$) compared with non-IRP CMV+ individuals (25.2, $n=23$)

observations that infection with CMV can reduce prevailing levels of immunity to EBV (Khan et al. 2004) also support this hypothesis. Similarly to the CD8+ T-cell expansions, it has been shown for the CD4+ T-cells that the CMV-specific response expands considerably with age altering the CD4+ repertoire (Pourgheysari et al. 2007), and that VZV-specific populations (Fletcher et al. 2005) are significantly decreased when CMV-specific CD4+ cells expand.

Surprisingly, however, we found that among sero-positive individuals, the IRP individuals showed a significantly lower number of expanded clones than the non-IRP's (Reker-Hadrup et al. 2006, Fig. 3). We also found that a decrease in clone numbers among IRP individuals was associated with increases in the inflammatory activity by elevated plasma IL-6 as well as with shorter survival times. This suggests that increased numbers of clonal expansions is beneficial to the individual, indicating an increased clonal expansion diversity and immune protection capability. It also support the hypothesis that when a critical point is reached, clonal exhaustion leads to shrinkage of the clonal expansion repertoire, detrimental to immune capabilities both for unrelated antigens and for CMV itself (Reker-Hadrup et al. 2006).

4.2.6 Low-Grade-Inflammation

There is considerable evidence of age-associated changes in immune capabilities resulting in increased morbidity and mortality due to altered function of the innate immune system (Krabbe et al. 2004). Low grade inflammation increases in the level of the inflammatory markers TNF-α, IL-6, and CRP and decreases in the levels of albumin in plasma have been shown to be significant predictors of mortality in population studies in the elderly (Evrin et al. 2005; Bruunsgaard et al. 2003; Reuben et al. 2002). Many studies have focused on the multi-factorial cytokine IL-6 and suggest that ageing independently of any particular disease is associated with two- to four-fold low grade increases in the plasma levels of this inflammatory mediator. Studies have shown that low-grade increases in IL-6 levels are related to increased amounts of fat tissue and loss of muscle mass, strength, functional capability and weight that occur with normal ageing. CRP is considered as a surrogate marker of IL-6, because CRP is produced by IL-6 induction in the liver (Krabbe et al. 2004). Increases in IL-6 are also associated with many age-related diseases such as cardiovascular disease, arthritis, osteoporosis and Type-2 diabetes (Forsey et al. 2003), which represent major morbidity classes and causes of death in the very old.

Using data from the second and third waves of the NONA immune study, we were able to confirm results from other studies that have demonstrated that ageing is associated with low-grade inflammation and that inflammatory markers are significant predictors of mortality in the very old (Wikby et al. 2006, Table 4). Logistic regression analysis also revealed that the IRP and low-grade inflammatory activity, defined by the marker IL-6, were independently predictive of 4-year survival, an outcome that remained when CRP and albumin were entered as covariates (Wikby et al. 2006). The independent main effect predicted 57% of nonsurvival

Table 4 Inflammatory parameters in plasma at Time 2 in very old individuals that had survived (survivors) and not survived (non-survivors) at Time 3 of the NONA Immune Longitudinal Study

Parameter	Survivor	Non-survivor	p<
IL-6 (pg/ml)	4.9 (61)[a]	9.2 (21)	0.001
CRP (mg/ml)	1.4 (60)[a]	3.6 (22)	0.05

[a] Median (*n*)

and, impressively, 97% of survival, showing that IRP and IL-6 are better predictors of survival than of subsequent mortality. These parameters are consequently strong candidates as significant markers of healthy ageing. IRP and IL-6 were predictive of mortality and not significantly affected by eight prevalent diseases, including Alzheimer's, cardiovascular disease and Type-2 diabetes, controlling for age and gender (Wikby et al. 2006). These results are in agreement with findings demonstrating that low-grade inflammation (Krabbe et al. 2004) and IRP (Nilsson et al. 2003) can predict mortality independently of disease and comorbidity. While the IRP reflects changes in the adaptive T-cell system primarily associated with lifelong persistent CMV infection, the increases in IL-6 seem to reflect innate immune system changes, including a wide range of alterations associated with overall devitalisation and frailty. This is supported by our findings of changes in the plasma levels with decreases in albumin and increases in acute-phase proteins (Wikby et al. 2006).

The above results may at first seem contradictory to our baseline findings of elevated IL-6 plasma levels specifically associated with cognitive impairment and mortality. This association was not seen at second wave follow-up (Wikby et al. 2006). However, cognitively impaired individuals who survived until the follow-up or who became incident cases were more likely to be in their early stages of the disease process compared with those who showed manifest cognitive impairment already at baseline with higher subsequent mortality rates. Thus, it is likely that sample composition variously reflect reasons for survival or selective mortality in late life (Pawelec et al. 2005).

A comparison of the inflammatory markers IL-6 and CRP at baseline, and two years later (wave 2) for IRP survivors and nonsurvivors four years after baseline, was performed in the NONA study. The result demonstrated only a minor inflammatory activity in the subgroups at baseline, indicating that the IRP is not associated with inflammation per se (Wikby et al. 2006). Increases in the inflammatory activity found between baseline and wave 2 among nonsurvivors, however, show that IRP individuals develop such an activity by increases in IL-6 and CRP in a terminal decline stage (Wikby et al. 2006). The results suggest a linkage between adaptive T-cell and innate immune system changes for IRP individuals that begins with acquisition of CMV infection in earlier life and is followed by an expansion and accumulation of senescent CD3+CD8+CD28-T-cells, the development of an IRP and finally an activation of the innate immune system in a terminal decline stage late in life (Wikby et al. 2006), including low-grade inflammatory processes with the secretion of proinflammatory cytokines like IL-6 and TNF-α (Zanni et al. 2003).

4.2.7 IRP Movement

In the NONA Immune Longitudinal study only 5 individuals (4%) moved into the category at risk by changes in the CD4/CD8 ratio, which was a significantly lower percentage as compared to the previous OCTO Immune Study (30%). Intriguingly and contrary to findings in the OCTO Immune Study, however, we have found that a few NONA individuals (n=3) actually moved out of the IRP category (Wikby et al. 2006). The changes found were associated with increases in IL-6, IL-10, neutrocytosis and lymphopenia, suggesting that IL-6 may induce an antiinflammatory rather than a proinflammatory effect in association with enhanced IL-10, neutrocytosis and lymphopenia to limit the potential injurious effects of sustained inflammation in these particular and rare individuals (Steensberg et al. 2003).

4.2.8 Longitudinal Changes

To follow a population of very old individuals over time in a longitudinal study offers unique opportunities to examine intraindividual changes as well as to test various factors predictive of longevity. Throughout the 20th century a remarkable increase in lifespan has taken place in humans and the increased number of centenarians in recent decades is considered to mainly be due to a dramatic decline in the mortality rate among those above 80-years of age (Jeune 2002), that is individuals exclusive focused upon in our studies. There is evidence that infectious disease become more important in the very old and that the immune system thus may be considered decisive for successful ageing and longevity in humans (Delarosa et al. 2006). In the NONA Immune

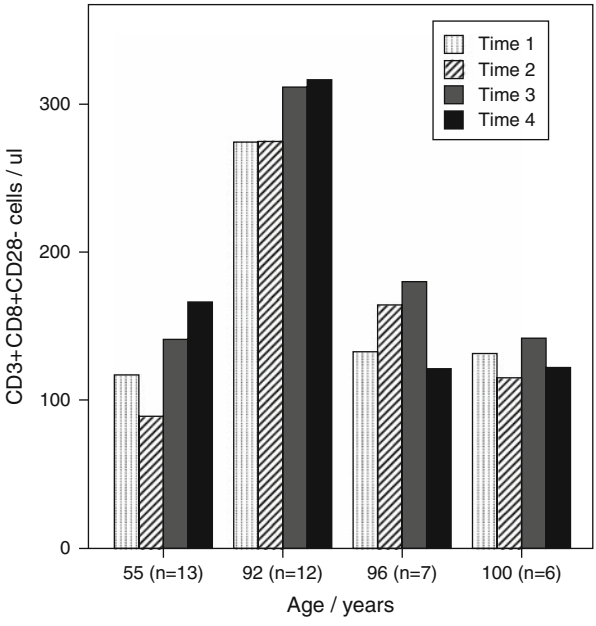

Fig. 4 Longitudinal data for the number of CD3+CD8+CD28- cells in subgroups of middle-aged and very old surviving through time 1 (1999), time 2 (2001), time 3 (2003) and time 4 (2005). Multivariate analysis of variance indicated significant differences between the age groups (p<0.05) with greater number of CD3+CD8+CD28-cells for the 92 year group as compared with other age groups. The analysis showed no significant change across time or group by time interaction effects

study, considering that the oldest cohort had become centenarians, commonly taken as a paradigm for "successful ageing" a question of significant interest was weather the "successfully aged" might be exceptional in their avoidance of the IRP.

Blood was drawn at baseline from 138 individuals with 42 belonging to the oldest 94-year old cohort, 47 to the 90-year cohort and 49 to the 86-year cohort. After 6 years, 99 individuals (72%) were deceased and another 8 declined to participate at this forth wave, giving a total number of 31 participants for the 6-year follow-up study. At baseline, 22 individuals resided in the IRP category and none of those had survived at 6-year follow-up. During the 6 year longitudinal study, five individuals developed an IRP by increases in the number of CD8+ and decreases in the number of CD4+ cells. Of these 4 were deceased at the 6-year follow-up, leaving only one individual with an IRP at 6-year follow-up (Strindhall et al. 2007).

At the 6-year follow-up, significant cross-sectional differences were found in the various T-cell subsets as well as in the CD4/CD8 ratio between age groups, differences not seen at baseline (Strindhall et al. 2007). The results suggest age-related changes but longitudinal data, however, revealed no significant changes at all across the 6-year period in any of the T-cell subsets (Figs. 4 and 5). These findings support the interpretation that the observed differences in the 6-year cross-sectional mean values are an effect of selective mortality. Individuals surviving until the age of 100 years did not display any T-cell changes associated with the Immune Risk Profile, i.e. they retain low numbers of CD8+CD28-cells and high CD4/CD8 ratio (Figs. 4 and 5), also predominant when these "successfully aged" people were younger, while among ten cases close to the CD4/CD8 cut-off of 1.00 (range 0.8–1.6), nine (including the one single IRP individual) belonged to the youngest age group (92 years old), and one to the 96 year old group (Strindhall et al. 2007). An effect of selective mortality is also

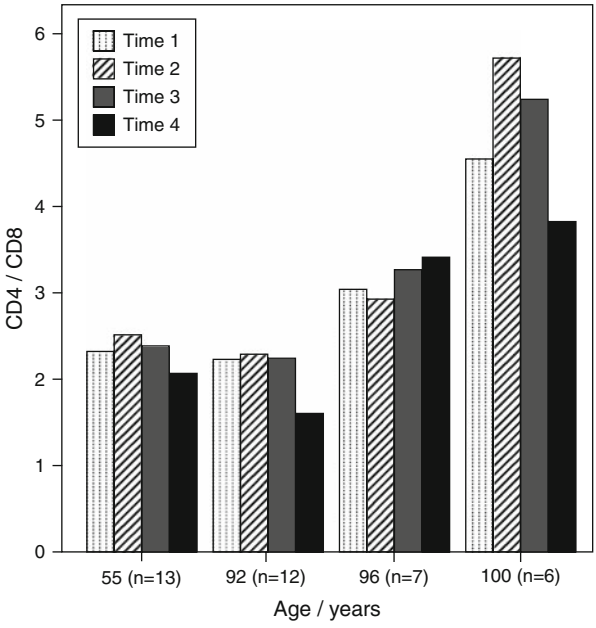

Fig. 5 Longitudinal data for the CD4/CD8 ratio in subgroups of middle-aged and very old surviving through time 1 (1999), time 2 (2001), time 3 (2003) and time 4 (2005). Multivariate analysis of variance indicated significant differences between the age groups ($p<0.05$) with the 96 and 100 year groups indicating higher ratio as compared with other age groups. The analysis showed no significant change across time or group by time interaction effects

supported by the fact that the prevalence of IRP decline from 16% at baseline to 3% at 6-year follow-up, when individuals in the NONA sample had become 95 years old on average.

The results also support the view that centenarians, although being "successfully survivors", they are not healthy (Jeune 2002). In the NONA Immune sample three quarters of the individuals were in fact classified as frail and at most 5% conformed to the SENIEUR criteria for being quite healthy (Wikby et al. 2006). The IRP, however, was shown to be predictive of mortality independently of the health status of the very old (Nilsson et al. 2003) and the absence of an IRP in centenarians therefore indicate a well preserved adaptive immune system, that helps to account for their survival in spite of substantial morbidity and co-morbidity.

5 Conclusions and Future Direction

Immunosenscence is the term used to describe the acquired dysfunctional immunity in old people and is characterized by changes in the T-lymphocyte system in particular. The changes become manifest as increasing numbers of lately differentiated T-cells that previously was exposed to antigens (memory and effector cells), and a decreasing number of cells being able to recognise and combat new antigens (naïve cells) that invade the human body (Akbar and Fletcher 2005). In the OCTO and NONA studies we have identified and examined a T-cellular IRP showing the above outlined characteristics of *immunosenscence*, i.e. the accumulation of dysfunctional terminally differentiated CD8+ cells with a CD3+CD8+CD27-CD28-CD45RA+CCR7-perforin+ phenotype and the depletion of the number of CD8+CCR7+CD45RA+ naïve cells (Wikby et al. 2005). Extensive analysis to search for associations between this IRP and various parameters including the psychosocial domains of physical and mental health, cognitive functioning, personal control/coping, social networks and everyday functioning capacity, clinical laboratory parameters, various diagnosed diseases and medication revealed that the IRP was associated only with evidence of persistent CMV infection (with EBV as a bystander). This result may indicate that CMV has a more insidious impact on the immune system than previously believed and also compared with other herpes viruses examined in these studies. The accumulation of large numbers of CMV-specific CD8+ T-cells as well as the finding that a majority of clonal expansions in the very old are associated with CMV has given additional information supporting the hypothesis that CMV greatly contribute to the development of an IRP and thus contributes to the development of *immunosenscence* in the elderly. Characteristics of the IRP identified in the OCTO and NONA studies are summarised in Table 5.

In the NONA Immune Longitudinal Study the IRP was studied in the context of low-grade inflammation, previously identified as a predictor of mortality in the old (Wikby et al. 2006). The IRP and low-grade inflammation were independently found to be main predictors of survival. This outcome was not significantly affected by individuals' health status, suggesting that the physiological ageing

Table 5 Characteristics of the Immune Risk Profile

Increased CD8+ and CD3+
Decreased CD4+ and CD19+
CD4/CD8 ratio < 1
Increased lately differentiated CD8+CD28-CD27- cells
Depletion of naïve CD8+CD45RA+CCR7+ cells
CMV-seropositivity
Clonal expansion of CD8+ cells carrying receptors for CMV
High proportion of dysfunctional cells among the CMV-specific CD8+ cells

processes of T-cell immunosenescence and low-grade inflammation are of crucial importance in late life survival (Wikby et al. 2006). The results also suggest a sequence of stages for IRP individuals (Fig. 6) that probably begins in early life with CMV infection, followed by the generation of large CD8+CD28-effector cell expansions to control lifelong persistent infection, homeostatic T-cell changes and a gradual change towards an IRP, that might be associated with a failure of the T-cell capability to control CMV. These individuals show decreased numbers of the CD8+ cell clonal expansions associated with increases in levels of plasma IL-6 and shorter survival, suggesting a stage in ageing where clonal exhaustion may lead to shrinkage of the clonal expansion repertoire detrimental to immune capabilities (Reker-Hadrup et al. 2006). It ends in a terminal decline stage with a low-grade inflammatory process that occurs in late life (Wikby et al. 2006,

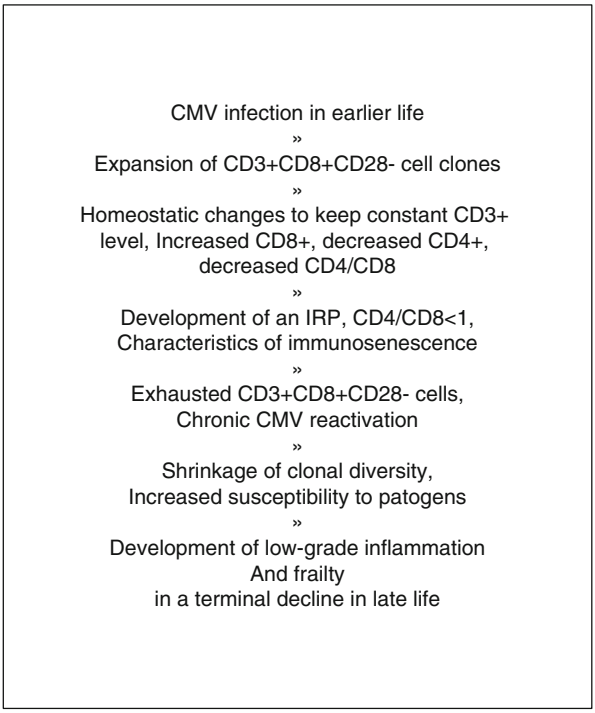

Fig. 6 Processes in a sequence of stages in the human life span of importance for late life survival in IRP individuals
The sequence is based on findings in the OCTO and NONA immune longitudinal studies and supports the inflamm-ageing hypothesis (Franceschi et al. 2000)

Fig. 6). This supports the inflamm-ageing hypothesis in human ageing suggesting that age-associated chronic inflammation causes frailty and that immunosenescence is driven by a chronic antigen load, associated with CMV infection, that induces a progressive expansion of compromised poorly functional CD8+CD28- effector T-cells (Franceschi et al. 2000; Fulop et al. 2005). The CD8+CD28- cells are able to secrete pro-inflammatory cytokines like IL-6 and TNF-a that may compensate for the defective T-cellular function, and/or amplify an ongoing inflammatory process (Zanni et al. 2003).

In future studies it will be important to investigate why only a certain fraction of CMV sero-positive individuals reside in or move into the category of risk. It is also urgent to further characterize those exceptional individuals that move out of the category of risk, allowing insight into clinical intervention approaches for those who remain in the IRP category until death. It is important to specifically study the phenomena of clonal expansion regarding frequencies and specificities of cells for various clones and to gain a better understanding of the nature of the link between CMV infection, phenotypic T-cell changes and changes in proinflammatory cytokines associated with the IRP. We should also study the relevance of the IRP more comprehensively in relation to age and gender. Future research also need to be multidisciplinary and include more detailed medical and biobehavioral evaluations of risk individuals to more fully understand the complex immune alterations that are associated with the major IRP marker.

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Lymphocytes Sub-Types and Functions in Centenarians as Models for Successful Ageing

Enrico Lugli, Leonarda Troiano, Marcello Pinti, Milena Nasi, Erika Roat, Roberta Ferraresi, Linda Bertoncelli, Lara Gibellini, Elisa Nemes and Andrea Cossarizza

Contents

Abstract: Several cell subsets participate to the immune response, and their close interplay is fundamental for the successful elimination of harmful pathogens. In addition, a tight regulation of the immune response has to occur in order to avoid excessive inflammation and potential autoreactivity towards self components. In the last years, the discovery and the characterization of new lymphocytes subsets, including regulatory T (Treg)-cells and Natural Killer T (NKT)-cells allowed a better understanding of how an effector immune response is induced and therefore down-modulated. During the ageing of the immune system, a process termed immunosenescence, these subsets undergo a profound remodelling, both in phenotype and function. In this chapter, we will describe the essential features of lymphocyte populations in centenarians and the differences that occur with unsuccessfully aged people.

A. Cossarizza (✉) · E. Lugli · L. Troiano · M. Pinti · M. Nasi · E. Roat · R. Ferraresi · L. Bertoncelli · L. Gibellini · E. Nemes
University of Modena and Reggio Emilia
Chair of Immunology, Department of Biomedical Sciences
Modena, 41100- Italy
Tel.: +39 059 2055415
Fax: +39 059 2055426
E-mail: cossarizza.andrea@unimore.it

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1 Introduction

The progressive lengthening of the mean life span and the consequent growth of the elderly population has focused the attention of the scientific community on human longevity. Aging is a complex process characterized by a general decline in physiological function with an increasing morbidity and mortality. The specific causes of aging are not known. Several studies suggest an association between changes in immune function and longevity, and indicate that the deterioration of the immune function, termed "immunosenescence", could be the cause of the increased susceptibility to cancer, autoimmune and infectious diseases which characterize elderly. However, a common bias in the studies on immunosenescence has been the confusion between ageing and age-related diseases and the difficulty to study the immunology of the physiological ageing and not the immunology of the age-associated diseases. Centenarians have been proposed as model to study immunosenescence in physiological conditions, being exceptional individuals who have reached the extreme limit of human life escaping the major age-related diseases [1]. Many of them, the so called "healthy centenarians", have resulted free of diseases typical of ageing, such as cancer, dementia, diabetes, cardiovascular diseases and osteoporosis.

One of the most important characteristics of successful ageing is the ability to fight efficiently infective agents. An efficient immune response requires the coordinated action of several components, and is mainly due the presence of a consistent number of continuously renewed T- and B-cells that are equipped with a clonotypic receptor recognizing virtually every potential antigen. The immune system must have the ability to expand efficiently the adequate antigen-specific clone(s) and the ability of producing and maintaining memory cells that, during a following infection by a pathogen that has been recognized in the past, mount a more efficient response.

Immunosenescence is characterized not only by a simple deterioration of the functionality of the immune system, but also by a complex modification of several components. As a result, some immune parameters tend to diminish with ageing, while some others remain constant or even increase [1]. At the cellular level, features of immunosenescence are the constant decline in the number of naïve T-cells, the reduction of new B-cell precursors, and the tendency to expansion of T- and B-clones in the periphery, reflecting in a diminished capacity to recognize antigens [2, 3]. Indeed, at the molecular level, the expansion of antigen-specific clones is paralleled by a restriction of the T-cell repertoire, that defines the amplitude and diversity of the molecules that form the T-cell receptor (TCR) [4]. This is also accompanied by the restriction of the B-cell repertoire, and indeed the presence of clonal B-cell expansions that give origin to monoclonal gammopathies is relatively common in aged individuals, accompanied by a decline in peripheral blood B-cell count [5, 6].

2 An Overview on the Immune System

In order to cope with all possible antigens that can be encountered in the course of human life, T-cells have the capacity to generate theoretically 10^{15} different TCR, that form a really large T-cell repertoire. It has been estimated that, in a young healthy adult, about 10^8 different TCR are present in every moment [7]. T-cell compartment is generated and maintained by the production and output of new T-lymphocytes, naïve for their antigen, from the thymus. Such production tends to decline with age of about two orders of magnitude, and is considered the leading force of immunological ageing. Thymic activity is extremely efficient during childhood, but very low in the elderly. This is likely due to the fact that the immune system has to cope very early with an environment full of infectious agents, and thus has to be extremely strong and maximally functional in the first period of life, *i.e.*, during childhood.

In parallel with the decline of thymic acticvity, an increase in the number of circulating memory T-cells exists during ageing because of the differentiation and maturation of naïve T-cells, and/or the expansion and maintenance of memory cells that continuously encounter the same (persistent or recurrent) antigen. The contribution of these two components to the circulating T-cell pool changes with age. As thymic output declines (while the number of possible encounters with infective agents obviously increases with age), the relative importance of the reexpansion of "old" but experienced memory cells becomes more relevant than the differentiation of naïve T-cells. This age-related accumulation of memory cells can represent a response to the reduced number of naïve T-cells, required to fill the so-called "immunological space", or conversely a cumulative effect of the expansion of cells, likely due to persistent, subclinical infections [8–10]. It is not still clear which is the precise dynamics of the functional decline of the immune system, and at which age the generation of T-cells in the thymus is eventually exhausted. Some authors, based on the rate of reduction of the thymopoietic tissue, have estimated a complete loss of thymopoiesis at 105 years [11], but this estimation was clashed by the observation that active thymic tissue can be found even later [12]. Moreover, a recent study revealed the existence of a second organ that produces T-cells in mice, but this evidence still lacks in humans [13].

The pool of naïve T-cells can be maintained throughout life by a mechanism called "homeostatic proliferation", induced by cytokines such as interleukin (IL)-7 and IL-15. Small amounts of these molecules can maintain a small rate of T-cell proliferation, and thus keep the system alerted. After stimulation with these cytokines, naïve T-cells from elderly subjects can show a reduced capability to differentiate and proliferate. This seems to indicate that naïve T-cells that have undergone homeostatic proliferation are not fully functional, probably because an intrinsic reduction of their proliferative potential, and so a full immunological response cannot be generated [14].

The role of another key population, that of "naturally occurring" CD4+ regulatory T-cells (Treg), is currently under analysis. This is a population of T-cells with suppressor capacity, that regulate a wide variety of immune responses [15–18], including the activity of self-reactive T-cells that can potentially cause autoimmune disease. Treg exert their suppressive function in different manner, either by contact or production of inhibitory molecules, and preferentially express high levels of CD25 (the low affinity chain of the IL-2 receptor), the winged-helix family transcription factor forkhead box P3 (FoxP3) [18], the ectoenzymes CD39 and CD73 [19–21], and lack the interleukin-7 receptor α-chain (CD127) [22, 23]. Controversial data exist on the role and amount of this cell subset with age, and it is unclear whether and how these cells are altered, or in some way related to the immune dysfunction in the elderly [24]. It has been reported that the thymic output of Tregs may decrease when there is a significant loss of its capacity to generate new T-cells, and thus the homeostasis of Tregs has to be sustained by alternative pathways, *i.e.* the generation of Tregs in the periphery [25, 26]. Scanty data actually exist on this aspect of immune regulation, and further studies are needed.

The occurrence of modifications in the production and release of growth factors (such as G-CSF, SCF) or interleukins (such as IL-2, IL-7, IL-9, IL-13, IL-15) and chemokines (such as CXCL12, sCXCL10 and sCCL2) has been described either in the thymus or in the periphery, along with changes in the production of haematopoietic cells and of other components, including cells forming the microenvironment where lymphocytes and monocytes are produced and activated. The cytokine network undergoes profound modifications with age, and several authors have shown the relevance of such a phenomenon [27–32].

Centenarians provide the best example of successful ageing and are an excellent model to understand the complex modifications of the aforementioned processes. They are exceptional individuals who have reached the age of 100 years in a relatively good state of health, from many points of view (cognitive, physical, endocrinological, biochemical and immunological) [33–35]. Studies on their immune system have revealed parameters that follow the degenerative trend often present in aged people (eg, reduction of B- and T-lymphocytes, reduction of proliferative capability), whereas other parameters are well preserved (natural killer cell activity, chemotaxis, phagocytosis) or even increased (production of proinflammatory cytokines) [33, 34]. In this chapter we will discuss the main features of lymphocyte subsets from centenarians in order to identify an "immunological signature" which is responsible for their difference with the entire elderly population.

3 B-cells in Centenarians

During ageing several changes in the B-cell compartment, in terms of new B-cell generation, homeostasis, repertoire and functionality, can occur. Peripheral B-cells and their progenitors can be classified in different subsets on the basis of phenotypic, anatomic and functional parameters. Most B-cells originate from bone

marrow, where common lymphoid precursors are committed to specific lineage commitment, with rearrangement of immunoglobulin (Ig) genes and subsequent expression of surface IgM (sIgM). After B lineage commitment, cells rearrange the Ig heavy chain genes in a stage defined "pro-B-cell". Successful rearrangement initiates pre-B-cell stage, where cells express a pre-B-cell receptor (BCR) together with Igα and Igβ transmembrane signalling molecules. After a brief proliferation, the Ig light chain genes are rearranged, and cells express a complete surface receptor, defined as the BCR, characteristic of immature B-cells.

Immature B-cells complete their differentiation in the periphery, in a series of stages collectively defined as "transitional stages", classified as T1, T2 and T3 on the basis of surface expression markers. Cells that successfully complete differentiation join to peripheral pools; the large majority becomes mature follicular B-cells (the so-called B2-cells), which include precursors of primary antibody forming cells as well as memory cells, and represent more than 80% of B-lymphocytes [36]. Others cells join the marginal zone pool of lymph nodes, where they play a major role in response to T-cell independent antigens, or in the very early phase of T-cell dependent response. Even if the exact mechanism driving the differentiation in follicular or marginal zone B-cells is not fully clear, it is clear that BCR signal strength plays a crucial role in such a process [37, 38].

The last compartment of B-cells is formed by B1-cells (mostly CD5+), the first that appear during development, which is maintained by self renewal. B1-cells were originally identified as CD5+ B-cells participating in autoimmunity, and sharing similarities with those causing human chronic lymphocytic leukaemia [39, 40]. In humans, B1-cells are normally about 1–5% of the total B-cells, and are found in a variety of tissues including the spleen, peritoneal cavity, pleural cavity and intestines. B1-cells can be further divided in B1a or B1b using surface markers CD19, CD45 (B220), and CD5. B1a-cells are CD19+, CD45+ and expresses high levels of CD5, while B1b are CD19+, CD45+ and express low to almost-absent levels of CD5 [41].

Concerning naïve and memory B-cell subpopulations, a series of studies have shown that human B-cell subpopulations can be distinguished on the basis of CD27 expression and have striking characteristic features [42–45]. In particular, it is possible to identify three main subsets: CD19+, IgD+, CD27- (naïve B-cells), CD19+, IgD+, CD27+ (memory cells that underwent somatic hypermutation, and express high affinity IgM), and CD19+, IgD-, CD27+ (memory cells that switched Ig class) [44, 45].

The expression of CD27 on B-cells increases gradually with age: cord blood B-cells do not express CD27, whereas approximately 40% of adult peripheral blood B-cells are CD27+ [46, 47]. These two subpopulations are different: indeed, CD27+ B-cells are large cells with abundant cytoplasm, whereas CD27- B-cells are smaller and have a scanty cytoplasm [48].

Studies on the B-cell compartment in centenarians were not as accurate as those regarding T-cells. As in the case of T-cells, it is widely accepted that the maintenance and renewal of the B-cell pools are subjected to a complex network of homeostatic processes which undergoes to substantial modifications with ageing. During the

‘90s, several studies have shown a significant modification in the pattern of B-cells subpopulations (reviewed in [33, 34]). It was shown that the proportion of B-cells in the peripheral blood usually decreases in elderly persons, including centenarians [6]. Moreover, age-related increase of the serum level of immunoglobulin classes (IgG and IgA but not IgM) and IgG subclasses (IgG1, 2 and 3, but not IgG4) was detected [49].

Conversely, less attention was paid to modifications of the B-cell compartment during ageing, and only a few studies have analyzed B-cells subsets in centenarians [50–52]. These studies have shown an age-dependent decrease in the absolute number of CD5+ and CD40+ B-cells, and a slight, even if not significant decrease of CD19+, CD27+ cells. The changes in absolute counts were mainly due to the decrease of the absolute number of B-cells. The percentage of CD19+, CD27+ B-cells increased significantly with age, reflecting increase in memory cells and decrease in naïve B-cells; centenarians did not escape from this trend. It was observed that the percentage of IgD+, CD27+ memory cells increases until 30–40 years, and then declines, with a secondary deficiency in IgM production in elderly subjects. The shift observed towards memory cells, as in the case of T-cells, can mirror the continuous exposure to foreign antigens throughout life [50, 51].

Similar results were obtained by other authors, who analyzed changes in B-cells with ageing, in a population of healthy subjects 21-99 years old, and demonstrated a rapid increase in the absolute number of memory B-cells (either IgD+ or IgD-) in the first three decades of life, and then a slight decrease of IgD-, CD27+ B-cells, and a marked decrease of IgD+, CD27+ elements. Concerning the percentage of these subset among B cells, CD27+ B cells increase during childhood and adulthood and then decline, the most marked decline regarding IgD+, CD27+ cells. The percentage of naïve B-cells increased with age. Again, extremely old people fit perfectly the trend observed in “normal” people [52]. Functional studies have shown that memory B-cells in the elderly have remarkable diminished production of Igs after stimulation, and that induction of plasma cell differentiation was decreased in elderly persons compared with that in adults [52]. These observations are in complete agreement with the reduction of clonotypic response to new antigens, accompanied by the progressive expansion of monoclonal B-lymphocytes observed in the elderly and the consequent increase in monoclonal immunoglobulin (MIg).

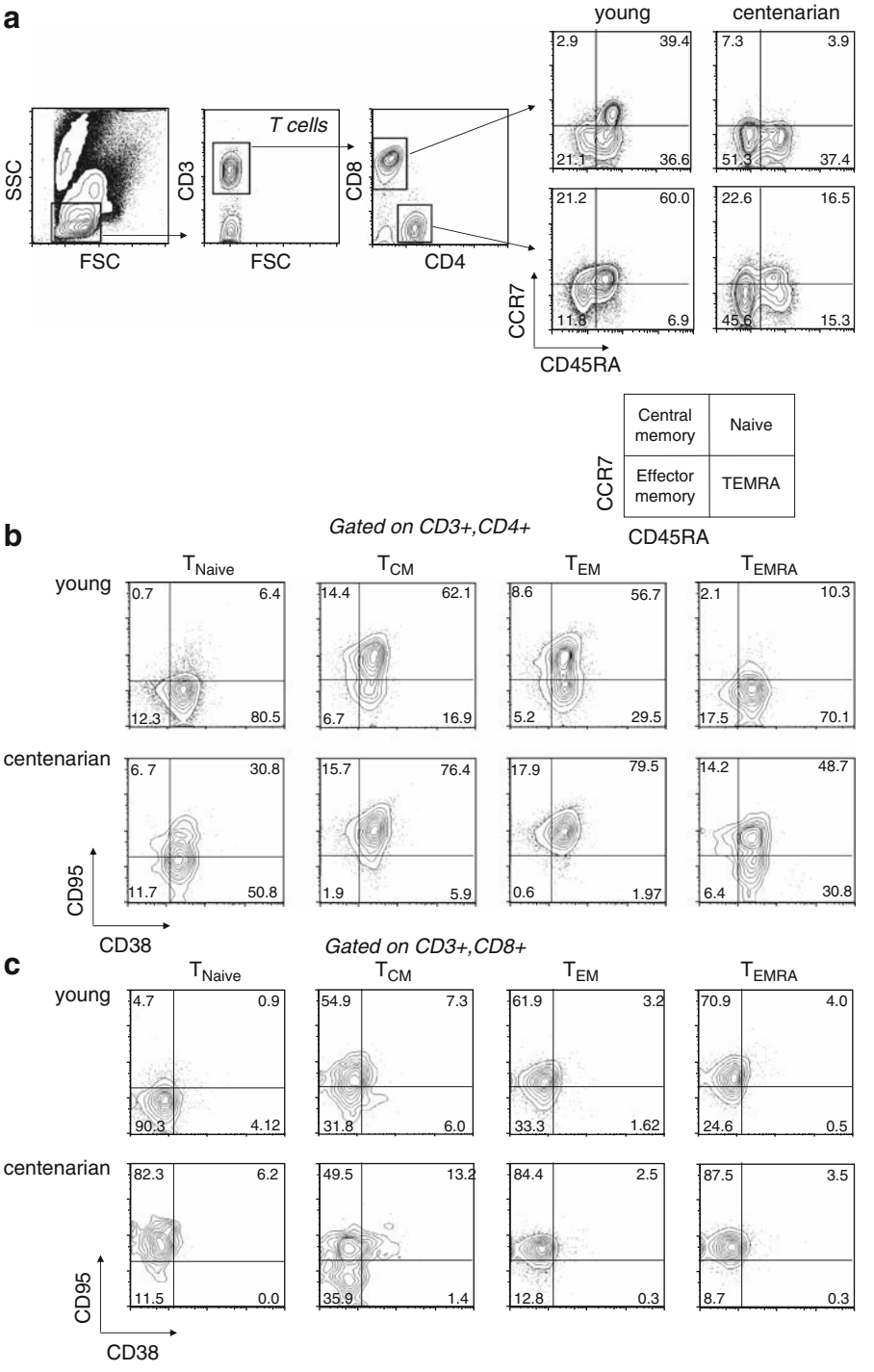
MIgs are known to appear with a high frequency during ageing and indeed about 20% of elderly humans have serum MIg; there are direct evidences that, in the mouse model, cells producing MIg derive from expansion of single clones [53]. About 1% of elderly subjects transform these alterations into myeloma, and perhaps chronic lymphocytic leukaemia, a lymphoid malignancy that appears with a relative frequency in advanced age [54]. The shift in the specificity of antibodies from foreign to autoantigens observed with ageing is mirrored by the specificities of serum MIg, according to data indicating that approximately 50% of MIg reacts with autoantigens [55]. Indirect evidence from studies performed in mice suggests that cells that secrete MIg derive from the CD5+ B-cell population, even if also CD5- monoclonal B-lymphocytes can be present in humans [56].

Such modifications of B-cell subsets with ageing, and in particular the progressive increase in memory B-cells and the reduced capability to cope with new antigens, as well as with recurrent encounters with the same antigen, is clearly reflected in deep changes in the production of antibodies. The concentration of natural and antigen-induced antibodies specific for foreign antigens decline with age, as well as specific antibody responses to almost all vaccines [57]. Despite this defect in the antibody response to foreign antigens, the level of serum Ig does not decline during ageing [57], a paradox that can be explained by an age-dependent increased serum concentration of autoantibodies [58]. Thus, ageing is associated with alterations in the B-cell repertoire with respect to the ratio of antibodies specific for the nominal versus self antigen. The autoantibodies detected at increased concentrations in the serum of elderly people are specific for autoantigens such as DNA, immunoglobulins, thyroglobulin, and are found at high concentrations in patients with systemic lupus erythematosis, rheumatoid arthritis or hypothyroidism. However, it is interesting to note that centenarians are characterized by a striking absence of organ specific autoantibodies, whereas nonorgan specific autoantibodies increase in healthy aged donors, as well as in centenarians [6, 33, 34, 59].

The serum concentration of IgM, IgA and IgG also increases with age [60] although the concentration of IgD decreases in elderly people, including centenarians [61]. The preferential loss during ageing of IgG and high affinity antibody, the most protective antibodies against bacterial and viral diseases, can be related to the increased susceptibility and severity of infections and a lower efficacy of vaccines in elderly people.

4 General Features of T-cells in Centenarians

The fine analysis of the phenotype of peripheral T-lymphocytes is crucial for a better comprehension of the T-cell homeostasis during ageing. Not only this allows to determine T-cell dynamics, but also to deeply investigate the role of specific T-cell subsets. One of the most age-related changes within the T-cell population is the progressive accumulation of memory cells in spite of the naïve T-cell pool [62–65]. It is to note that in the past years several studies, including ours, have used the expression of CD45 isoforms, CD45RA and CD45R0, to define naïve/unprimed and memory/experienced T-cells, respectively [62]. As a consequence, it was reported that a well preserved number of naïve T-cells can be still present in people with advanced age, included centenarians [33, 62]. Few years later the publication of such studies it was shown that CD45RA+ cell population was quite heterogeneous, includes terminally differentiated T-cells, and that several different memory subsets are present in the peripheral blood, which can be recognized by the simultaneous use of anti-CD45RA, anti-CCR7 and anti-CD62L monoclonal antibodies [66]. Interestingly, a particular subset of memory cells, the so-called TEMRA (T effector memory RA+) subset, more frequent in the CD8+ than in the CD4+ compartment, is formed by terminally differentiated memory cells that are capable of reexpressing the CD45RA


a
young
centenarian
SSC
FSC
CD3
T cells
FSC
CD8
CD4
2.9
39.4
21.1
36.6
7.3
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51.3
37.4
21.2
60.0
11.8
6.9
22.6
16.5
45.6
15.3
CCR7
CD45RA
Central memory
Naive
Effector memory
TEMRA
CCR7
CD45RA
b
Gated on CD3+,CD4+
T Naive
T CM
T EM
T EMRA
young
centenarian
0.7
6.4
12.3
80.5
14.4
62.1
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16.9
8.6
56.7
5.2
29.5
2.1
10.3
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70.1
6. 7
30.8
11.7
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76.4
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14.2
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CD95
CD38
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Gated on CD3+,CD8+
T Naive
T CM
T EM
T EMRA
young
centenarian
4.7
0.9
90.3
4.12
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31.8
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61.9
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0.0
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13.2
35.9
1.4
84.4
2.5
12.8
0.3
87.5
3.5
8.7
0.3
CD95
CD38

isoform, but are incapable of recirculating in secondary lymphoid organs. These CD45RA+ revertant cells have been definitely demonstrated to behave as memory cells [67].

The advent of polychromatic flow cytometry (PFC) has increased the capacity to analyze several antigens in the same cell and, as a consequence, has allowed a better definition of T-cell differentiation state. As evidenced in Figs. 1 and 2, multiple subsets can be identified in the peripheral blood by the simultaneous analysis of differentiation (*i.e.* CD45RA, CCR7, CD95), activation (*i.e.* CD38) and survival (*i.e.* CD127) markers. PFC led to demonstrate that the use of only one or two markers is not sufficient for the definition of naïve T-cell [68, 69]. PFC has been recently used by our group to analyze T-cell differentiation in centenarians, and we have found that in these subjects true naïve T-cells are extremely rare [70]. Indeed, a small proportion of CD4+ and CD8+ T-cells coexpress CD45RA and CCR7 [71], but further analysis of these "naïve" T-cells reveals that most of them also express CD95 (typically present on memory cells). Several questions still await an answer, such as where do these cells come from and where are they going (in terms of which lymphoid site is their final destination), do they represent an intermediate subset between naïve and memory cells, or are they terminal effector cells with the capability to recirculate to lymphnodes and spleen. It is to note that, in the elderly, the majority of CD45RA+ cells lack the costimulatory molecules CD27 and CD28 [64, 72], express CD57 and KLRG1 [73, 74] and produces IFN-γ upon stimulation [64], suggesting that these cells are part of the memory pool.

Both repeated exposure to antigens for more than a century and reduced thymopoiesis can account for the striking accumulation of memory T-cells in centenarians. It has been estimated that thymopoiesis declines over 80% after the age of 60 years and minimal or no thymic activity can be predicted after 100 years, due to the progressive loss of thymic epithelial space [75]. T-cell receptor rearrengement excision circles (TRECs), which are indicative of thymic activity [76], are practically undetectable in these subjects, although in our experience about 15% of centenarians (4 out of 25) displayed detectable levels of TREC+ lymphocytes [71]. It is thus likely that external factors such as the activity of homeostatic cytokines could contribute to the maintanance of the few naïve T-cells in old age [75]. However, competition with memory cells for those factors could compromise naïve T-cell survival and maintenance (see below).

IL-7 and IL-15 have been widely described as important cytokines for the regulation of T-cell homeostasis [77]. In particular, IL-7 plays a pivotal role in determin-

Fig. 1 Polychromatic flow cytometric analysis of peripheral blood T-cells from a centenarian and a young donor (23 years old) **(a)** Lymphocytes were first gated on the basis of forward (FSC) and side (SSC) scatter, then T-cell subsets were selected by gating on CD3+, CD4+ or CD3+, CD8+ cells. Further analysis of the expression of CD45RA and CCR7 allowed the identification of naïve (TN: CD45RA+, CCR7+), central memory (TCM: CD45RA-,CCR7+), effector memory (TEM: CD45RA-,CCR7-) and CD45RA+ terminal effector (TEMRA: CD45RA+, CCR7-) cells (b, c) Analysis of the expression of CD38 and CD95 in naïve and memory subsets of (b) CD4+ and (c) CD8+ T lymphocytes. Numbers indicate the percentages of the population identified by anti-CD38 and anti-CD95 mAbs

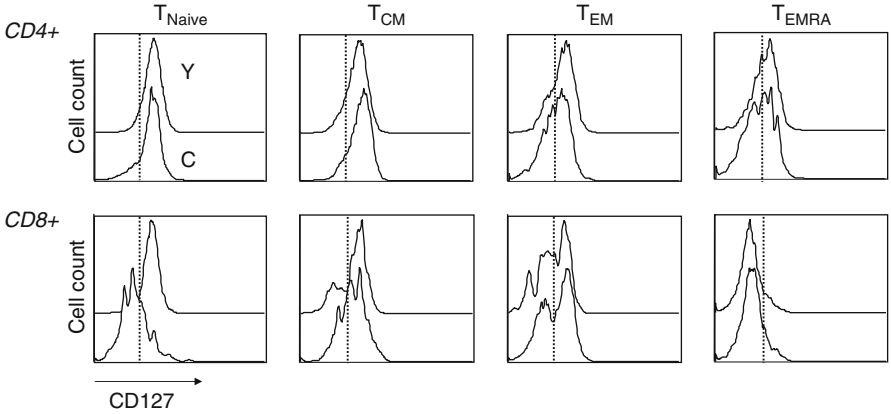

Fig. 2 Analysis of CD127 expression on naïve and memory subsets of CD4+ (upper panels) and CD8+ (lower panels) T-cells In each panel, upper histogram indicates CD127 expression in a young donor (Y), while lower panels are referred to a centenarian (C)

ing the survival of naïve T-cells and their proliferation in lymphopenic conditions. For naïve cells, IL-15 seems to be less important, since IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$ mice display only slightly reduced levels of naïve CD8+ T-cells, which could be due to modest defects in thymic production, or to effects on the survival and/or proliferation of naïve CD8+ T-cells [77–79]. It is noteworthy that CD4+ T-cells, which express higher levels of IL-7Rα than CD8+ T-cells, appear to be more dependent on survival signals mediated by IL-7 signals. IL-7 is also important for the maintenance of memory CD4+ T-cells while memory CD8+ T-cells mainly rely on IL-15 signals. Recently it has been reported that CD8+ T_{EMRA} cells from elderly subjects (>65 years old) display altered expression of CD127 and reduced responsiveness to IL-7 in vitro [80]. Differently, we recently demonstrated that centenarians do not undergo a remodeling of the IL-7/IL-7 receptor system, as it is in the elderly, suggesting an active role for this cytokine in very old age [71]. We found that plasma IL-7 levels were unmodified throughout life, and the same was observed for CD127, both at the mRNA and protein level [71]. However, more detailed analysis of T-cell subsets by PFC revealed that slight modifications regarding CD127 expression can be found in certain subsets after analysis of T-cell flow cytometric profile by novel bioinformatic approaches (see below) [70].

IL-15 may also play a role in regulating T-cell homeostasis in centenarians. IL-15 level is increased in these subjects [81] and may itself contribute to the accumulation of memory cells. IL-15 has also a potent capacity to induce peripheral T-cell expansion and, together with IL-7, may compensate loss of thymic activity by driving homeostatic turnover. The idea that aged subjects are characterized by a higher T-cell turnover than young subjects is supported by several experimental evidences: i) old people have nearly twice Ki67+ T-cells in the periphery in comparison to young donors [82]; ii) studies using the deuterated glucose technique revealed an accelerated turnover of the CD8+,CD45RA+ subset in the elderly population [83]; these cells, that in old people are probably part of the so-called T_{EMRA} subset, can

be generated, at least in vitro, by the proliferation of central memory T (T_{CM}) cells in response to hoemostatic cytokines rather than by the direct expansion of T_{EMRA} cells themselves [84]; iii) TREC+ cells, which are not only influenced by thimic activity but also by the rate of immune activation and proliferation occurring in the periphery, are undetectable in most centenarians [71]. As a consequence, it is possible to speculate that the expanded memory pool, which is a predominant feature of centenarians, may compete with naïve T-cells for IL-7 and IL-15 availability, thus limiting naïve T-cell survival and proliferation.

5 T-cell Function in Centenarians

There is large agreement that T-cell function is in part compromised during ageing, and it affects both CD4+ and CD8+ T-cells at the level of antigen-specific immunity [85]. These alterations regard many aspects of cellular function such as proliferation, intracellular signalling, cytokine production and effector function.

CD4+ T-cells display age-related reduced helper capability. Studies in aged mice revealed that effector CD4+ T-cells generated from naïve CD4+ T-cells are characterized by a reduced expression of differentiation and activation markers such as the CD40 ligand (CD154) and CD25 [86, 87]. As a consequence, reduced B-cells response was observed due to a defective helper activity [87], which is mainly ascribed to reduced IL-2 production [86]. Decreased production of IL-2 and impaired response to this cytokine have been documented in aged humans as well. However, altered production and utilization of this cytokine can be potentiated by exposing cells from aged donors to low frequency-pulsed electromagnetic fields, suggesting that these altereations are reversible and can be positively modulated [88].

Age-related defects in naïve CD4+ T-cells are likely due to the chronologic age of the CD4+ T-cells rather than to the chronologic age of the individual [14]. In fact, newly generated naïve CD4+ T-cells in old mice exhibit normal effector function *ex vivo* and *in vivo*. These data indicate that long-term maintenance of the naïve T-cell pool by homeostatic mechanisms may result in the alteration of T-cell activity. On the other side, restoring or boosting thymic activity may help in reducing immune defects in the elderly.

In addition to IL-2, alterations in the production of several cytokines have been detected such as decreased production of IL-4 by CD4+ T-cells from aged mice after stimulation with anti-CD3 antibody [89], or increased production of TNF-α from aged humans after stimulation with PMA/ionomycin [90]. By contrast, the production of TNF-α was not modified in centenarians [90]. Increased inflammation exerted by CD4+ T-cells could reflect the proinflammatory status which is often observed in the elderly but not in centenarians [10].

Reduced effector function has also been observed for CD8+ T-cells by analyzing antigen-specific immune responses. For example, CMV-specific CD8+ T-cells from >65 year old people are highly expanded in CMV carriers but they are impaired in IFN-γ and IL-10 production after CMV stimulation [91]. By contrast, secretion

of IFN-γ was observed after stimulation with mitogens. Surface receptor analysis revealed a highly differentiated effector-memory (CD45RA- CCR7-) or terminal effector (CD45RA+ CCR7-) phenotype and lack of CD28 and CD27 molecules but high levels of the KLRG-1 receptor, which is associated to end-stage differentiation and apoptosis resistance [91]. The same authors reported that CMV-specific CD8+ T-cells from HLA-A2+ centenarians were not so highly expanded and displayed lower KLRG-1 expression, suggesting earlier differentiation and normal mechanisms of apoptosis [91]. However, other authors reported that CMV-specific CD8+ T-cells for a HLA-B7-restricted epitope can occur at very high frequency in centenarians as well [92]. Differently, EBV-specific CD8+ T-cells response remains constant with ageing but it is interesting to note that in CMV-seronegative donors, the response to EBV increases significantly with age [93].

Modifications in the proliferation of peripheral blood lymphocytes (PBLs) from centenarians have been detected but they were not unidirectional. In fact, full capability of PBL proliferation has been observed in response to anti-CD3 antibody, pokewood mitogen and phorbol esters, while proliferation in response to PHA, IL-2, autologous and allogenic mixed lymphocyte reaction is reduced [94]. However, it is to be noted that, after PHA stimulation, PBLs from centenarians showed a delayed peak of thymidine incorporation but the overall thymidine incorporation was comparable to that of young donors [95]. Analysis of telomeres revealed an inverse correlation between age and telomere length, indicating that centenarians do not escape the phenomenon of telomere erosion. Interestingly, in fibroblasts from centenarians telomere length is indistinguishable from those from young donors [96, 97]. Thus the general idea is that lymphocyte proliferation in centenarians is in part preserved. As defects in the production of and response to IL-2 have been proven in human ageing, by contrast lymphocytes from centenarians are fully capable of binding IL-2 [33]. IL-2 could certainly sustain lymphocyte proliferation but it remains to be determined whether IL-2 production is critically modified in these subjects. Genetic analysis of the IL-2 promoter revealed that the IL-2 high-producer genotype is less frequent in centenarians than in young people [98]. These data contrast with what has been reported above but it is to be noted that an increase of IL-2 production characterizes the Alzheimer's disease serum profile. Moreover, people carrying the IL-2 low-producer genotype have a lower CD8 cell count in comparison to those carrying the IL-2 high-producer genotype. These data together suggest that the genetic background could not be a bystander factor in determining the so-called "immune risk phenotype" (IRP). Longitudinal studies identified the IRP phenotype as a composition of parameters which includes CMV seropositivity, a CD4:CD8 T-cell ratio of <1 due to increased CD8+ T-cells, an expansion of CD8+ CD28- T-cells with features of terminally differentiated T-cells, the presence of CD8+ T-cell clonal expansions, and elevated levels of proinflammatory cytokines in serum [99–101]. It has been also demonstrated that the IRP strongly influences the survival of people above the age of 80 [99, 100].

CD8+ T-cell clonal expansion is very common in aged people and has been also reported in animals [102]. In humans, a strong correlation exists between

age and the incidence of CD8+ T-cell clonal expansions, with one-third of adults over the age of 65 years developing CD8+ clonal expansions [103]. However, the occurrence of such a high number of monoclonal CD8+ T-cells does not seem to be pathological since CD8+ T-cell lymphomas do not develop in these subjects, suggesting that CD8+ T-cell clonal expansion is still under homeostatic control. Antigen may play a predominant role in the occurrence and maintenance of this phenomenon. In particular, CMV infection seems to drive CD8+ T-cell clonal expansion. By using MHC tetramers bearing CMV antigen, authors found that a T-cell clone specific for a single CMV antigen can account for a high proportion of the entire CD8+ T-cell pool in the elderly population [93, 104]; however, at the moment, it is still unclear whether this occurs also in the centenarian cohort [91, 92]. Persistent CMV infection is thought to actively contribute in the definition of the IRP; however, little is known on how CMV strongly influence subjects' survival in advanced age and how clonal expansion of CMV-specific CD8+ T-cells is driven, as these subjects did not display any reactivation of CMV infection [92]. Some authors hypothesized that such a high oligoclonal expansion of CMV-specific T-cells in the elderly population may shape the T-cell repertoire, fill the immunological space and compete for survival and growth factors [105]. For this reason, memory CD8+ T-cells specific for other antigens than CMV could be impaired [93] or lost through competition, resulting in the exposure of elderly people to otherwise silent infections.

Compared with a sample of very old, the prevalence of IRP and the associated increase of CMV specific T-cells might decline in a sample of centenarians by selective mortality, because survival in those aged 80–95 years occurs preferably in the non-IRP individuals [10].

6 Regulatory T-cells in Centenarians

It was supposed, since many years, that effector immune response should be tightly regulated in order to avoid excessive inflammation and, subsequently, tissue damage. This hypothesis and further experimental evidences suggested the existence of a subset of cells involved in the suppression of the immune response. Extensive research in the past decades led to the identification of suppressor T-cells as subsets of the CD4+ T-cell lineage. In particular, in 1995, Sakaguchi and colleagues reported that "activated" CD4+ CD25+ T-cells, now defined naturally-occurring regulatory T (Treg) cells, were able to maintain immunologic self-tolerance [106] while, in 1997, Roncarolo and colleagues identified an inducible subset of CD4+ T-cells capable of suppressor function [107]. These cells, therefore defined Type-1 T-regulatory cells 1 (Tr1), differed from Treg cells because they were not naturally present in the circulation but could be induced by prolonged treatment with IL-10 in vitro and responded after recognition of cognate antigen [107]. Further research confirmed that Treg cells originated from the thymus and constitute a different lineage from Tr1 cells and conventional CD4+ T-cells [108]. Treg cells constitutively

express the forkhead box transcription factor FoxP3, which acts as a key control gene of their development and function [109]. Differently, Tr1-cells and other subsets of suppressor/regulatory T-cells later identified, such T-helper Type-3 (T_H3) cells, are inducible and can develop from conventional CD4+ T-cells when exposed to specific stimulatory conditions such as the blockade of costimulatory signals, deactivating cytokines or different drugs [110].

Naturally occurring Treg cells constitute the 1-8% of total CD4+ cells in healthy adults. This large imprecision in the determination of their number could be due to the different criteria used for their identification (defined either CD4+, $CD25^{high}$ or CD4+, $CD25^{high}$, FoxP3+) or to the limited number of subjects studied. In fact, the definition of Treg cells solely based on the expression of high levels of CD25 (CD4+, $CD25^{high}$) could overestimate their number, since the CD25 antigen is also upregulated in activated T-cells. This raises several doubts on the reliability of CD25 as a unique marker of CD4+ Treg cells, expecially in the contest of chronic immune activation, such as HIV infection, autoimmune diseases and ageing itself, where an increased number of activated T-cells in the peripheral blood has been described [33]. Additional markers, possibly in combination and in the same cells, should be investigated for this purpose, such as FoxP3 [109], CD127 [22, 23] or CD39 and CD71 [19–21].

Whether the amount of Treg cells in peripheral blood is dependent on age is still a matter of debate. Many independent groups studied large cohort of subjects and positive correlations between age and the number of Treg cells were reported or not [24]. However, people with advanced age (>80 years) were considered only in a few studies and none of them were centenarians. Thus, up to now, no data are present on the number and function of Treg cells and other regulatory T-cell subsets in centenarians. Adding to this, controversial data are available on the influence of the ageing process on the function of Treg cells. A study reported the decline in the suppressive function of Treg cells by almost 90% with age over 50 years [111], but others reported equivalent function of Treg cells between young and old donors [112, 113].

Animal studies suggest that phenotipic and functional modifications can occur in this subset with ageing. In aged mice, high accumulation of CD4+, CD25+, FoxP3+ Treg cells has been observed in the spleen [114, 115] and lymph nodes [114], and these cells retained suppressive capability [114, 115]. Removing these cells by anti-CD25 monoclonal antibodies restored effector CTL response and anti-tumour immunity [114]. However, major suppressor activity was found in a subset of CD4+, CD25- cells [116], which have been later demonstrated to harbour intracellular FoxP3 [115]. Thus, it is possible that other subsets rather than only CD4+, $CD25^{high}$ cells are able to regulate effector responses. It remains to be determined whether these CD25- suppressor cells were $CD25^{high}$ Treg cells in origin, or have been generated from conventional CD4+ T-cells under particular conditions of stimulation. In any case, further experiments, including the analysis of multiple Treg markers together with functional studies, are required to clarify the role of the ageing process on this CD4+ lineage.

7 γδ T-cells in Centenarians

In addition to conventional αβ T-cells in blood and in peripheral tissues, a second subset of T-cells bearing a different T-cell receptor, composed of γ and δ chains, can be identified. These γδ T-cells represent only 5% of total T-cells in peripheral blood but are enriched in many organs containing epithelia such as skin, lung, intestine, and genitourinary tract [117]. Since multiple γ and δ genes are available, different combinations of γ and δ chains are possible, thus generating different families of γδ T-cells. Intriguingly, γδ T-cells in different epithelial tissues use distinct Vγ/Vδ chains; for example, in the intestinal epithelium and lamina propria, γδ T-cells, which represent 30% and 5% of total T-cells, respectively, are mostly Vγ8/Vδ1, while in the peripheral blood Vγ9/Vδ2 are found [117]. These data suggest that different subsets of γδ T-cells may recognize specific antigens and may play different roles during the immune response.

Despite a strong similarity with conventional αβ T-cells dictated by the presence of a TCR and αβ surface markers, γδ T-cells exibit cytolitic activity by a major histocompatibility complex (MHC) antigen-unrestricted mechanism [118]. Thus, γδ T-cells are not activated by peptides presented by antigen-presenting cells (APC) but by nonpeptidic compounds of low molecular weight and cell-cell contact is needed for γδ T-cell activation to occur [117, 119, 120]. So far, their most potent activator is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the microbial nonmevalonate pathway of isopentenyl pyrophosphate (IPP) biosynthesis [121, 122]. Other ligands than phosphoantigens can be recognized by γδ T-cells, including MHC-class I like molecules, such as T10 and T22 in mice and MICA and MICB in humans, and an ATP synthase F1-apolipoprotein A-I (AS-ApoA-I) complex [123]. The role of these cells in immunity is still to be clarified but γδ T-cell response is nowadays considered foundamental in tumor surveillance and in infectious diseases.

In several microbial infections in humans, such as tularemia, salmonellosis, brucellosis and ehrlichiosis, γδ T-cells are expanded up to 48-97% of total T-cells [124]. Increased levels of circulating γδ T-cells have been also described in infections with protozoal parasites (malaria, toxoplasmosis, leishmaniasis) and mycobacteria (*M. avium* and *M. tuberculosis*) [124]. Recent studies in nonhuman primate models concerning the major subset of γδ T-cells, *i.e.,* that expressing the Vγ9/Vδ2 TCR, revealed that γδ T-cells may play an active role during the early phases of the immune response [125]. In macaques, the expansion of γδ T-cells was detected 2–3 weeks after inoculation of *M. Bovis* BCG, and was observed in the lung and intestine, but not in lymphoid organs [125]. Accumulation of γδ T-cells at infection sites but not in lymph nodes has been also described in murine infections. Moreover, γδ T-cells specific for the murine MHC class Ib molecule T22 harboured Vγ4 and Vγ1 in the spleen and Vγ7 in the gut epithelium [126]. These data together suggest that this lymphocyte population acts locally in a tissue-specific, and not antigen-specific, manner and is excluded from secondary lymphoid organs.

However, contrasting data have been reported. CCR7 and CD62L receptors, which are able to mediate the homing to secondary lymphoid tissues, are not expressed on the majority of Vγ9/Vδ2 T-cells. The phenotype of these cells resembles that of conventional effector T-cells, *i.e.* CD45RA-, CD45R0+, CD27-, CD11a^{bright} [127], which are preferentially localized to nonlymphoid tissues [128]. However, upon activation with cognate ligand, γδ T-cells can acquire an APC phenotype, by inducing the expression of HLA-DR, CD80 and CD86 costimulatory molecules together with CCR7. These cells are able to present antigens to conventional αβ T-cells, thereby activating the adaptive immune response [117, 129].

Due to the difficulty to obtain specimens from different anatomic sites, the majority of studies conducted in humans concerns peripheral blood γδ T-cells. Recent data reported a prominent role of these lymphocytes in regulating intestinal homeostasis [120], and thus it would be interesting to check whether this sort of protection is maintained in the elderly or, if altered, could be responsible for immune pathologies of the gastro-intestinal tract.

Different subsets of γδ T-cells are affected by age in a different way. In fact, while the absolute number and percentage of Vδ2 T-cells progressively diminishes with age, that of Vδ1 remains rather constant throughout life [130, 131]. This obviously leads to a subversion in the Vδ2/ Vδ1 *ratio*, that is more prominent in centenarians than in old donors, despite the total γδ T-cell count does not differ between the two groups [130]. Ageing did not change the proportion of γδ T-cells as regards to αβ CD3+ T-cells, suggesting common mechanisms of depletion [130]. It is thus possible that thymic involution and peripheral expansion may play a role in regulating the homeostatis of γδ T-cells. However, these aspects need further elucidation.

Interestingly, γδ T-cells were not impaired in their cytolitic potential in old age, despite an age-dependent decrease in proliferative capability in response to isopentenyl diphosphate (IPP), which was completely ascribed to the Vδ2 subset [130]. Increased production of TNF-α, but not IFN-γ, by γδ T-cells has been observed in centenarians in comparison to young donors [130]; moreover, γδ T-cells from centenarians displayed higher tendency to undergo apoptosis after treatment with TNF-α and anti-CD95 monoclonal antibody [131]. Higher percentage of CD95+ γδ T-cells in centenarians may reflect the accumulation of effector memory-like T-cells, which are known to be highly sensitive to activation-induced cell death mediated by signals passing through CD95/Fas. Milder alterations in the γδ T-cell population have also been described in old people [130, 131]. This suggests that a progressive loss of γδ T-cell activity is observed with age and, as for αβ T-cells, centenarians do not escape this phenomenon.

Whether γδ T-cells play a role during immune responses in old age is still a matter of debate. Many papers confirmed a continued, protective role of this subset in adult animals [132, 133], including humans [134–136] but recent studies in mice revealed a largely redundant role in the presence of a fully mature and expanded αβ T-cell compartment [137].

Sex-dependent phenotypic and functional differences have been described in γδ T-cells. In particular, the number of V 9/V 2 cells and their effector capacity remain constant with age in females, while drop in males [138]. It would be inter-

esting to confirm these data in old people and centenarians in order to determine whether this cell subtype could influence the female predominance in the centenarian population.

8 NK-cells in Centenarians

NK-cells are lymphocytes that can recognize and kill virus-infected as well tumor cells without antigen-presentation or MHC-restriction. NK and T-cells share a common precursor, that expresses FcγRIII, but can develop independently of the presence of the thymus, as shown in athymic mice [139]. NK-cells do not rearrange immunoglobulin (Ig) or T-cell receptor (TCR) genes and therefore neither Ig nor the TCR/CD3 complex is expressed at the cell surface, except for the ζ chain [140]. In humans, these cells are characterized by the expression of CD56, an isoform of the neural cell adhesion molecule (N-CAM), CD16, the low-affinity IgG Fc receptor (FcgRIII-A), CD57, an oligosaccharide antigenic determinant, and CD2, an adhesion molecule that appears to be correlated with the acquisition of Fas ligand-mediated cytotoxicity [141]. They also express inhibitory receptors that interact with MHC class I molecules and prevent unwanted destruction of the target cells. Thus the function of NK-cells results from a balance between activating and inhibitory signals delivered by specific membrane receptors and NK cell activation requires the interaction of activating NK receptors with their ligands on the targets and also the lack of inhibitory signals initiated by the interaction of NK inhibitory receptors with target MHC class I molecules.

They can kill target cells by the secretion of specialized lysosomes, containing pore-forming protein perforins [142], or by the induction of programmed cell-death pathways [143]. Some cytokines (such as IL-2, IL-12, IL-15, IL-18 and IFN-α/β) can induce NK cell proliferation and activation, migration and production of IFN-γ, TNF-α and GM-CSF (for review see [144]). NK-cells can produce also cytokines and chemokines that directly participate in the elimination of pathogens or activate other cellular components of immunity.

Several alterations have been described in NK cell function with ageing both in animals and humans. In humans, the different selection criteria of the elderly populations have produced contrasting data. Some authors reported a decrease in cytotoxic function of the circulating NK-cells of elderly subjects [145] which is associated with an increased incidence of infectious diseases [146, 147]. Indeed, there is an increase of mortality risk of 3 times in people more than 85 years with low numbers of NK-cells respect those with high NK cell numbers [148]. Several pathologies usually associated with ageing are associated with low NK cell activity in the elderly, such as atherosclerosis [149]. On the contrary, high NK cytotoxicity is associated with lower incidence of infections of the respiratory tract and with a better development of protective antibody in response to influenza vaccination [150].

Indeed, in centenarians, NK cell number, as revealed by analysis of CD56 expression on PBMCs, and functionality are not modified in comparison to young

donors while a partial loss of NK cell cytotoxic activity can be found in middle-age subjects, despite the CD57+ and CD16+ populations, which are capable of rapid cytotoxic activity, increased as in centenarians [151]. Further studies revealed a preferential expansion of the terminally differentiated $CD56^{dim}CD16+$ subset while minor modifications were found in the $CD56^{bright}CD16-$ subset [152].

Whereas NK cell activation mediated by CD16 is not affected by aging [144, 148, 149, 153], poor data exist on the function of other NK receptors and probably other NK activating or inhibitory receptors are defective in the elderly. It was reported that the expression of HLA-specific killer receptors is not significantly affected in NK-cells from elderly [154], but more recent studies have shown that NK-cells display an age-related increase in KIR expression and a reciprocal decrease in CD94/ NKG2A expression, although the CD94/NKG2A inhibitory signaling pathway is intact [153].

The killing activity mediated by perforins is not modified in people with advanced age and no significant decrement of these molecules has been observed in NK-cells from young and old donors [155]. Interestingly, a greater decline of perforin expression is present in elderly men if compared to elderly women [156]. This could be a further element to understand the typically higher percentage of females among centenarians [157].

NK-cell activity and phenotype can be affected by the differential presence of cytokines between the young and the old population, as well as centenarians. In fact, a reduced production of cytokines involved in NK-cell activation, i.e, IL-2, IL-12, IFN-α and IFN-γ, is observed with increased age [158]. Accordingly, IFN-γ secretion by NK-cells in response to IL-2 [159] and chemokine secretion in response to IL-12 or IL-2 decrease in elderly [160]. In aged mice and humans the response of NK-cells to IFN-α/β is decreased and could be related to the delay in virus clearance observed in aged mice [159]. The decrease of NK-cell secretion could lead to an impaired adaptive immune response that could contribute to age-related diseases.

The functionality of the immune system is strongly influenced by the presence of certain hormones in a circuit that is called “neuroendocrine immune system” [161], which undergoes profound remodelling with increasing age [162]. NK-cells, as other immune cells, express some hormone receptors on their surface. As a consequence, certain hormones of the hypothalamic–pituitary–gonadal axis as well as thyroid hormones, dehydroepiandrosterone (DHEA), insulin-like growth factor (IGF)-1, melatonin or insulin regulate their function. Moccheggiani *et al.* showed that hormonal treatments with T3, T4, melatonin, GH or IGF-1 in old mice can restore NK-cell cytotoxicity and IL-2 and IFN-γ production [163, 164]. In healthy nonagenarians and centenarians, NK cell number and/or cytolytic activity was positively associated with serum levels of vitamin D, while T3 and i-PTH hormones were associated only with NK-cell number, suggesting a positive role of these molecules in regulating NK-cell homeostasis [165]. Preserved NK-cell functionality in these subjects, *i.e.,* NK-cell cytotoxicity and IFN-γ production, was also associated with good zinc ion bioavailability which, by contrast, is reduced in old animals and humans, but not in centenarians [165–167].

9 NKT-cells in Centenarians

The term "NKT" includes more than one subset of T-lymphocytes that have different phenotype, functional capacities and tissue distribution and that express NK-associated receptors (NKR) [168], historically CD161 in humans [169] and NK1.1 in mice [170].

The large number of studies regards the so-called "classical" or "invariant" NKT-cells (iNKT) expressing a semi-invariant T-cell receptors (TCR), characterized in most cases by Vα14/Vβ8.2 in mice [171] and by Vα24/Vβ11 in humans [172, 173]. iNKT-cells TCR can recognize CD1d (a monomorphic class Ib molecole) [174] and bind endogenous glycosphingolipids and α-glycuronosylceramide (present on the microbial cell wall), suggesting a role in the protection from bacteria that are not detected by classical pattern recognition receptors [175–178].

iNKT-cells can be divided at least in three subset on the basis of the expression of CD4 or CD8 coreceptor and on their cytokine production. iNKT-cells that express CD4+ produce Th1 and Th2 cytokines, while CD4- NKT-cells primarily produce Th1 cytokines. CD4- NKT-cells can be further divided into CD4-CD8- (double negative; DN) and CD8+ NKT-cells, which predominantly express the CD8αα dimer instead of the CD8αβ form present on conventional cytotoxic T lymphocytes [179–181]. Indeed, it has been shown that iNKT-cells express CD45RO but lack CD62L. It was hypothesized that this effector memory phenotype probably derives from the endogenous self ligands recognition [182]. A minority of iNKT-cells are classified as central memory (CCR7+CD45RO+), while most Vα24CD4+ and CD4- NKT-cells could be defined as effector memory cells (CCR7-CD45RO+)[183].

There is evidence that CD1-restricted NKT-cells represents a thymus-dependent population. They are absent in nude mice, do not develop in thymectomized mice and first appear in the thymus slightly later than most other T-cell subsets. There is also convincing evidence that NKT-cells segregate from conventional T-cells at the stage of double positive (CD4+CD8+, DP) thymocyte in the thymic cortex [184]. Indeed, it seems that they acquire a relative resistance to activation-induced apoptosis in the late stage of intrathymic development [185].

iNKT-cells play an important role in host defense and immunoregulation, including the prevention of tumor development and metastasis, suppression of allergic responses and protection against viruses, parasites, bacteria and their products [171, 173, 186]. The most striking property of NKT-cells is their capacity to secrete large amounts of cytokines (IFN-γ, IL-4, IL-2, IL-5, IL-10, IL-13, GM-CSF and TNF-α) within minutes after TCR stimulation. Activation of NKT-cells also leads to upregulation of CD40L, resulting in IL-12 production by dendritic cells upon CD40 triggering [187]. Upon TCR engagement, NKT-cells have cytotoxic activities through the release of perforins and granzymes and by the expression of membrane-bound members of the TNF family (such as FasL) [188]. Activation of NKT-cells leads to subsequent activation of other cells, such as NK-cells, B cells, DC, macrophages, and conventional T-cells in mice as well as humans [189, 190]. Thus, they can affect the acquired immune system by activating pathogen-specific

CD4 Th1 cells as well as CD8 T-cells, suggesting an important role in conferring protection against microbial pathogens, like malaria. Moreover, they have been shown to play a crucial role in interfering with the initiation, growth and metastatic spread of tumours. NKT-cell-derived Th2 cytokines, such as IL-4, can downregulate immune responses and have been shown to contribute to protection against the development of autoimmune diseases (reviewed in [191]). Consequently, NKT-cell activation results in a cascade of immune reactions, providing a possible explanation for their regulatory effects.

Human iNKT-cells can be identified either by their invariant TCR formed by Vα24 and Vβ11 gene segments, or by CD1d-tetramers loaded with α-galactosylceramide (α-GalCer), a marine sponge-derived glycolipid able to selectively activate iNKT-cells in a CD1d-dependent manner [171, 192]. In human peripheral blood, however, classical CD1d-restricted NKT-cells are typically less than 0.1%.

Besides iNKT-cells, a different subset of conventional CD1d-independent α/β T-cell, called "NKT-like" or "nonclassical NKT"-cells, can express several NKR, such as CD16, CD56, CD57, CD161, CD94, NKG2A. The majority of NKT-like-cells likely belongs to nonclassical subpopulation and are mostly CD8+. The nonclassical NKT-cells can account for 5-20% of total T-cells in human peripheral blood [193, 194].

A limited number of studies investigated the role of peripheral blood NKT-cells in aged people. Studies on iNKT frequency in peripheral blood of centenarians from Okinawa were performed by Miyaji *et al.* who analyzed the so-called "extrathymic T-cells", characterized by the expression of CD3 and CD56 or CD57. They found a higher frequency of these cells compared to middle-aged subjects but no differences were detected between males and females [194]. Thus, these authors confirmed that the proportion and the absolute number of NKT-cells (CD56+ or CD57+ T-cells expressing Vα24+) were highly increased in the blood of centenarians, along with the proportion of IFNγ-producing cells among NKT-cells [166].

Other studies showed an effect of age on the homeostasis and function of circulating NKT-cells but elderly subjects rather than centenarians were considered. DelaRosa et al. found a decreased percentage of Vα24+ T-cells in elderly when compared with young controls and, within Vα24+ T-cells, a significant increase in the percentage of Vα24+CD4−CD8+ T-cells, while the percentage of Vα24+ within CD3+CD28+ was similar [195]. In accordance with that study, an age-related decrease in the percentage and absolute count of Vα24+Vβ11+ iNKT-cells has been shown in healthy individuals, although their functional capacity to respond to α-GalCer was not altered [196]. Indeed, a decline of 3.4% per year was evaluated in Vα24+Vβ11+ iNKT-cells with age [197], which involved both their absolute levels and their proportion as to the total T-cell compartment. In addition, they found a gender-related difference in the frequency of circulating iNKT-cells, that was lower in males than in females, and decreased faster with age in the formers than in the latters [197]. Similar results were obtained by Peralbo *et al.* who also reported a decreased proliferative potential of Vα24+Vβ11+ iNKT-cells in reponse to α-GalCer in healthy elderly compared to young subjects [198]. A decrease in the frequency of iNKT-cells in the elderly has been also reported by Jing *et al.* which

was also associated with an alteration in the iNKT-cell subset compositions, that is an increase in the proportion of the CD4(+) subset and a decrease in the proportion of the CD4/CD8 DN subset [199]. In addition, iNKT-cells from aged people produced predominantly Th2 rather than Th1 cytokines [199].

Whereas iNKT-cells are characterized by the expression of a semi-invariant TCR that interact with CD1d loaded with glycolipids, ''NKT-like'' T-cells are NKR-expressing conventional T-lymphocytes which display an oligoclonal TCR repertoire able to recognize classical MHC molecules loaded with peptides [200]. Most of NKT-like cells have an effector memory phenotype and contain high levels of perforin and granzymes [201]. NKR-expressing T-cells expand with aging and centenarians do not escape this phenomenon, as revealed by the increased expression of CD56 on T-cells [151].

Little is known about the function of NKT-like cells but the general belief is that their accumulation is primarily driven by a chronic inflammatory environment, as it is in the elderly population as well as in patients with persistent viral infections, rheumatic diseases and autoimmune diseases, in which a chronic stimulation of the immune system occurs [201]. In particular, the expansion of NKT-like cells accompanies the loss of CD28 expression on T-cells after antigenic stimulation in vitro and is associated with the accumation of $CD28^{null}$ T-cells *in vivo* [202].

In summary, several studies have demonstrated age-related effects on iNKT-cells, a diminished proliferative functionality, a shift from Th1 to Th2 response and a modification in the iNKT subset ratio. However, only one study investigated the frequency of these cells in centenarians.

All these findings may contribute to highlight the role of NKT in the general deterioration of the immune response in the elderly. Considering the importance of these cells in the recognition and elimination of Gram-negative bacteria [178], these defects could be involved in the increased morbidity and mortality due to bacterial infection associated to ageing. The age-dependent alterations in NKT-cells might also reflect the thymic involution, as conventional T-lymphocytes [203–205]. Molling *et al*. suggested that iNKT-cells decrease could affect an efficient tumor immunosurveillance in aged donors, representing a risk factor for tumour development [197]. Furthermore age-dependent alterations in iNKT cytokine production might contribute to the dysregulation of the cytokine network shown in the aged people [206, 207].

10 Bioinformatics Tools for the Analysis of Cellular Dynamics in Centenarians

As described above, a huge number of lymphocytes subsets exists in human peripheral blood. The fine analysis of these subtypes is of extreme importance for a better understanding of the cellular dynamics during physiological processes such as the ageing of the immune system. So far, the simultaneous analysis of multiple parameters at the level of single cell can only be performed by polychromatic flow

cytometry [208, 209]. A huge amount of data can be generated by such a technology which is, however, difficult to manage. In fact, several functional different populations can be identified by combining the positive and negative expression of each antigen (typically 2^n, where *n* is the number of parameters analyzed). Thus, using 8 fluorochromes coupled to 8 different monoclonal antibodies, it is thus possible to identify 256 lymphocyte subpopulations in 100 μL of blood.

T-cells from centenarians were recently studied by 8-colour flow cytometry in our laboratory. By combining the expression of CD45RA, CCR7, CD127 (IL-7rα), CD95 and CD38 (whose expression can be further distinguished between *dim* and *bright*), we were able to identify up to 48 subpopulations both for CD4+ and CD8+ T-cells (Fig. 1). In order to uncover subtle differences among the three groups of subjects under investigation (20 years old donors, middle aged and centenarians) that otherwise could be missed by classical approaches, we used global approaches based on the Cluster Analysis (CA) and on the Principal Component Analysis (PCA), which are often used for microarray experiments [70]. In particular, the former is able to generate groups or "clusters" of variables on the basis of their similarities and differences, while the latter allows the dimensionality of a multidimensional dataset to be reduced, in order to obtain a new system of coordinates, *i.e.* the principal components. In this ideal space, subjects are plotted by considering all the variables, *i.e.* the T-cell subsets generated by boolean combination. These analyzes revealed that, in centenarians, CD4+ T-cells can be highly heterogeneous since it was not possible to cluster centenarians on the basis of the CD4+ T-cell flow cytometric profile. PCA of CD4+ T-cell subsets revealed the expansion of either CD95+ central memory or effector memory cells where the expression of CD127 could be retained or not. A different behavior was observed for CD8+ T-cells, where a striking expression of terminally differentiated effector (CD45RA+, CCR7-) T-cells with a preferential CD95+, CD127-, CD38- phenotype was detected. More detailed analysis by using different approaches for data pretreatment, such as data scaling, revealed that, for instance, that the same memory subset from young donors and centenarians differentially express CD127. These data thus suggest that, while the production of IL-7 remains constant throughout life [71], T-cells subsets from centenarians could be differentially regulated in terms of peripheral homeostasis [14, 80, 105, 210].

These approaches are very useful to identify cellular dynamics during the ageing process and to identify minimal difference among different ages or clinical conditions. More detailed analysis, in particular in larger cohorts, will reasonably lead to the identification of specific subsets with a possible protective role towards diseases of various origins.

11 Concluding Remarks and Future Directions

We have described some crucial modifications occurring in different lymphocyte subsets with ageing, and underlined that centenarians display some special features that are not shared by the entire elderly populations. Whether these components,

rather than genetic or environmental determinants, are responsible for reaching such an advanced age still remains to be determined. It is however general opinion that all of the aforementioned factors act in synergy. Until now, many studies investigated whether the function of a specific subset is maintained or modified in these individuals, but data are lacking on the interplay among different lymphocyte populations.

An efficient immune response is the result of the tight cooperation of many cell types, and the disfunction of one of them can lead to the persistence of the antigen (or the pathogen), and to the onset of a chronic inflammatory enviroment. Thus, it is needed to uncover specific interactions among cell types by using more global approaches such as systems biology, genome-wide analysis and bioinformatics. A complete and detailed picture of the immune system of centenarians can reveal potential targets for therapy and vaccination in the elderly.

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Mouse Models and Genetics of Immunosenescence

Qing Yu, Jyoti Misra Sen and Dennis Taub

Contents

Abstract: Age-related changes in the immune system result in deterioration in the ability of elderly human beings to develop immunity after vaccination and to respond to infections. Thereby the quality of longer lifespan enjoyed by modern man is significantly compromised. Furthermore, higher mortality in the elderly from infections, autoimmune disease and cancer is associated with decline in the immune function. The use of rodent models has yielded critical knowledge of mechanisms by which immune cells develop and function. In this chapter, we focus on several mouse models that have provided significant data on the changes in immune system with advancing age. A greater understanding of many of the age-related changes in immune function, recently defined as immunosenescence, may provide important insight into the development of clinical strategies and interventions for the maintenance of adequate immune system as human beings age.

Keywords: Immune system • Thymic involution • Immune aging • Mouse models • Immunosenescence

1 Introduction

In mammals, adaptive immunity complements the more primitive innate immunity resulting in a more comprehensive protection from infection and neoplasms. Cells of the adaptive immune system, T-cells and B-cells are activated by the antigenic stimulation provided by the pathogen in combination with various growth factors and

D. Taub (✉) · Q. Yu · J. Misra Sen
Laboratory of Immunology
Clinical Immunology Section
National Institute of Ageing
Intramural Research Program
National Institute of Health
Baltimore, MD 21224-6825, USA
Tel: 410-558-8159; Fax: 410-558-8284
E-mail: taubd@grc.nia.nih.gov

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immunomodulatory molecules of the innate immune system (e.g., cytokines). The mammalian immune system undergoes significant changes throughout the animal's lifespan. Aging has been associated with immunological changes (immunosenescence) including thymic involution, lower number of naive T-cells, decrease in several cell immune functions and increase in others, and poor vaccination response to new antigens. At each stage of immune development over a lifespan, complex changes have been observed involving multiple cell types and molecular events, making it unlikely that we will be able attribute the age-related changes to a single gene or signalling pathway. Therefore, it has become imperative to understand not only the development and function of individual cell types that participate in normal immune responses and age-associated immunosenescence but also the interactions between various cells and signalling mediators and growth factors.

Many of the early efforts to examine age-associated immune dysfunction have centered on possible loss or alterations in the number of circulating lymphocytes, more specifically T-cells (Taub and Longo 2005). The focus on T-cells makes sense given the fact that T-cells are produced by the thymus, which is known to involute with advancing age resulting in a significant loss in its capability to generate new T-cells for export into the peripheral T-cell pool. Interestingly, this age-associated loss in thymic output does not result in any significant change in the total peripheral number of T-cells. It is believed that peripheral T-cell numbers with aging are maintained by a homeostatic compensatory process involving the peripheral thymus-independent expansion of mature T-cells. Given that T-cells have a limited replicative lifespan, the continued proliferation of T-cells with age is believed to lead to an accumulation of replicative-senescent T-cells possessing a diminished capacity to respond to new or recall antigens and activation stimuli. This homeostatic expansion of peripheral T-cells results in a significantly limited T-cell receptor (TCR) repertoire with age (Taub and Longo 2005). Moreover, while a number of additional age-associated alterations including effects on TCR and growth factor signaling, loss of bone marrow and thymic activity and output, alterations in cytokine and hormone expression and deficits in accessory cell function have been reported, the literature contains a number of contradictory findings describing age-related alterations in immune function suggesting significant variability in the immune aging process. Such variability hinders the identification of a central factor(s) responsible for the loss of immune function. Therefore, with the involvement of so many distinct processes within the aging immune system, the development of potential therapeutic interventions and strategies to reverse the aging process and rejuvenate the immune system has been hindered. Given that the loss in thymic function is one of the earliest and most consistent steps in the progression to immune dysfunction, strategies that target the involuting thymus are the focus of many interventions with the specific goal to reverse thymic atrophy and restore thymopoiesis and T-cell export. Increases in the numbers of new and functional T-cells in the circulating pool may extend and expand the peripheral T-cell repertoire as well as the individual's ability to mount a response to new or recall antigens.

In addition to the age-associated decline in T-cell function, the number of B-cells generated also decline with age resulting in a paucity of naive B-cells. Diminished immune responses to novel antigens in the elderly are the cumulative

effect of fewer newly generated naïve T- and B-cells and a decline in B-cell function and proliferative capacity. This decline in B-cell generation results from the deterioration of the hematopoietic stem cells (HSC) as well as the inability of the older bone marrow environment to support lymphopoiesis. This has lead to increasing interest in the study of HSCs and the local lymphoid environment and their ability to survive, expand and mature during the aging process. Moreover, apart from age-associated defects observed in the adaptive immune system, a number of immunological defects have also been observed in cells of the innate immune system including dendritic cells (DCs), natural killer (NK) cells, monocytes and macrophages, neutrophils, eosinophil, basophil and mast cells. The innate immune system forms the first line of defence in a host with phagocytes (macrophage, DC, granulocytes) engulfing microbes and particulate antigens, killing microbes and tumor cells and presenting processed antigens to T-cells. Defects in many of the established functions of innate immune cells have been reported (Taub and Longo 2005). Thus, it would appear that aging has an impact on many of the immune cell subsets and together may impact our ability to respond to antigens, microbial challenges and tumors with advancing age. Thus, increasing our understanding of the potential mechanisms believed responsible for age-related immunosenescence should yield valuable insight into the development and optimization of interventional strategies aimed at restoring thymic and bone marrow function and boosting the responsiveness of adaptive and innate cell subsets.

Animal models are commonly utilized in aging research to obtain data on specific tissue and organ systems that are difficult to obtain directly from humans. In addition, given the significantly shorter life span of animals and ethical issues with performing certain treatments in human volunteers or patients, it is much easier to examine the impact of genetic, hormonal, nutritional and physiological changes in these models than in humans. For example, one can easily examine the impact of caloric restriction in mice and rats over their 2–3 year life span compared to similar studies in human, which would be nearly impossible to perform from birth to death. Moreover, in mice, various genes can be manipulated and modified to generate gene hyper-expressing or deficient animals to examine the influence of a single gene pathway on life span or various physiological functions. A number of animal model systems have been established examining the age-associated defects in immune function and specific pathways that have been shown to be altered, accelerated and/or influenced by normal or pathological aging. These immunosenescence models include mice with alterations in telomerase activity, tumor suppressor function, oxidative stress, hormone expression and various other molecules associated with immune development and differentiation as well as longevity. A number of unique findings have been made in these models regarding basic immune function and the relationship between various pathways associated with longevity and immune function. Many of these models can be divided into several categories including those mouse models demonstrating a shorter life span, an extended lifespan and little to no significant differences in life expectancy. In this chapter, we have reviewed and consolidated the available data on many of these established mouse model systems that have been utilized to study alterations in innate and adaptive immune function and their relationship to age-related immunosenescence and clonal exhaustion.

2 Mouse Models Demonstrating a Shorter Life Span

A. Klotho Mice. Klotho gene is the first gene to be documented that accelerates aging and shortens life span upon disruption and extends life span when overexpressed (Kuro-o et al. 1997; Kurosu et al. 2005). The Klotho protein is a 130kD single-pass transmembrane protein, but the extracellular fraction of the protein is shed and secreted into the blood and body fluids and thus it is now believed to function as a hormone or cytokine. The Klotho protein inhibits intracellular insulin and IGF-1 signalling, which is a major mechanism for Klotho's effect on preventing aging (Kurosu et al. 2005). Klotho mice, which bear an insertional mutation of Klotho gene, have demonstrated various disorders resembling human aging as well as a significantly shortened life span (Kuro-o et al. 1997). Klotho mice have dramatically accelerated age-related thymic atrophy (Kuro-o et al. 1997) and significantly accelerated age-related decline in B-lymphopoiesis (Okada et al. 2000). The defective B-lymphopoiesis is not cell autonomous and thus Klotho may exert its effect by influencing hematopoietic microenvironment, which includes IL-7 gene expression by bone marrow stromal cells (Okada et al. 2000).

B. Senescence-accelerated Mouse (SAM). The SAM series were generated in 1980s from the breeding of AKR/J mice when the researchers found that certain litters of mice had an accelerated senescence in an inherited manner. To date, the SAM series includes nine SAMP strains with accelerated aging and three SAMR strains with normal aging, and each SAMP mouse shows various strain-specific and age-associated phenotypes (Higuchi, 1997; Hosono et al., 1997; Takeda et al., 1991; Takeda et al., 1981). The genetic changes in SAMP mice await extensive investigations, although alterations in the expression of apolipoprotein A-II (Apo A-II) has been identified in these strains (Higuchi 1997). SAMP mice demonstrate age-associated early decline in various immune functions, including decline of antibody production to T-independent antigens and NK cell activity; decline in antibody response to T-dependent antigens as a result of impaired T helper cell activity for antibody response and early onset of autoantibody production (Haruna et al. 1995; Hosokawa et al. 1987a; Hosokawa et al. 1987b; Hosono et al. 1997; Yoshioka et al. 1993).

C. Terc Deficient Mice. Telomerase is the protein complex that synthesizes telomeres and thus preventing telomere shortening during cell division and the consequent chromosomal instability and cell cycle arrest or apoptosis. Telomerase consists of two basic components, telomerase reverse transcriptase (Tert) and telomerase RNA component (Terc), which provides the RNA template for the telomeric DNA repeats (Collins 2000; Nugent and Lundblad 1998). Mice that are deficient for Terc are initially normal, but after 5–6 generations both females and males are sterile and demonstrate certain premature aging phenotypes and shorter life spans (Blasco et al. 1997; Lee et al. 1998). These mice also exhibit an impaired ability to regenerate hematopoietic cells after ablation of these cells by 5-FU, decreased antibody responses and germinal center formation to a T-dependent antigen and reduced T and B-cell proliferation after activation in vitro. These

results confirm that telomere shortening can influence immunoresponsiveness, although the precise mechanisms by which this occurs remains unknown (Herrera et al., 2000; Lee et al., 1998).

D. Tumor Suppressor Mouse Models. A number of genes that are involved in tumor suppression, DNA repair and cell cycle checkpoint also regulate aging and life span. The direct studies on effect of tumor suppressor P53 have been hampered by the early onset of tumor in $p53^{-/-}$ mice and failure of embryonic development in mice overexpressing p53 (Donehower et al. 1992; Tyner et al. 2002). However, mice expressing two types of truncated forms of p53, p24 and p44 (that bestow enhanced activity to endogenous full-length p53), show early onset of aging, a range of aging phenotypes and significantly decreased life span (Maier et al. 2004; Tyner et al. 2002). Disruption of Wip1, Brca1, Atm, Ku86, K70 or XPD all lead to shortened life span by modulating p53 activity (Barlow et al. 1996; Cao et al. 2003; Choi et al. 2002; de Boer et al. 2002; Li et al. 2007; Vogel et al. 1999). In $p53^{-/-}$ mice, apart from the development of thymic lymphoma, peripheral CD4+ T-cells demonstrate traits of immune senescence such as accumulation of memory type cells and defective proliferative response upon activation (Clarke et al. 1993; Donehower et al. 1992; Lowe et al. 1993; Ohkusu-Tsukada et al. 1999). In mice deficient for Wip1, a serine/threonine phosphatase that inhibits p53 activity, T-cell and B-cell proliferation response to mitogen stimulation is reduced and the immune system also manifest some other changes indicating of immune senescence (Choi et al. 2002). In $Ku86^{-/-}$, $K70^{-/-}$ and $Atm^{-/-}$ mice, development of T- and/or B-cells are severely impaired due to the critical requirement of these genes for recombination of antigen receptor genes and the incidence of lymphoma development is dramatically increased, both of which prevent a thorough study of the effect of these genes on immune aging (Gu et al. 1997; Li et al. 1998; Li et al. 2007; Matei et al. 2007; Matei et al. 2006; Nussenzweig et al. 1996; Zhu et al. 1996). The development and aging of the immune system in other interesting aging models, $p53^{+/m}$ mice, p44-Tg mice and TTD ($XPD^{-/-}$) mice remains to be thoroughly investigated.

E. Reactive Oxygen Models. Reactive oxygen species (ROS) are involved in pathology of aging and cancer and therefore molecules that provide defences against oxidative stress and ROS are important in preventing aging. Peroxiredoxin is a family of small antioxidant proteins that scavenge peroxide and play a role in the cellular response to ROS. Peroxiredoxin-$1^{-/-}$ mice have a shorter life span due to hemolytic anemia and develop several types of cancer including T- and B-cell lymphomas (Neumann et al. 2003). The mice have impaired innate immune systems with decreased number of NK-cells that express activation receptor Ly49D and decreased NK-cell cytolytic activity as well as reduced NK-enhancing activity in RBCs. The impaired NK-cell activity may be one of the factors responsible for increased tumor development in these mice (Neumann et al. 2003).

F. Hormonal Models. Growth hormone/insulin-like growth factor 1 (GH/IGF-1) signalling pathway has long been associated with the aging process and has been demonstrated to negatively affect life span, primarily by decreasing cellular antioxidative capacity and increasing cell apoptosis (Bartke 2005; Everitt 2003; Quarrie and Riabowol 2004). Transgenic mice expressing GH demonstrate various

Table 1 Mouse Models Demonstrating a Shorter Life Span

Strain name (reference)	Molecular description	Immune system	References (immune system)
Klotho mice (Kuro-o et al. 1997)	Mice with an insertional mutation of Klotho gene Novel membrane protein and aging suppressor, 20–40% sequence homology to bacteria and plant β-glucosidases and mammalian lactase glycosylceramidase. Function as a hormone and inhibits insulin and IGF1 signaling Multiple disorders resembling human aging Life span: decreased significantly	Significantly accelerated thymic atrophy with thymus normal early after birth but barely detectable at 6–9 weeks of age Markedly decreased B-cells in bone marrow, spleen and blood Reduced IL-7 responsive B-cell precursors Decreased IL-7 gene expression in bone marrow stromal cells but injection of IL-7 does not rescue the defective B-lymphopoiesis HSCs have normal capacity of B-lymphopoiesis in vitro and in normal hosts Normal B-cell development in neonates and young KO mice before 2 weeks of age that have no aging phenotypes	(Kuro-o et al. 1997) (Okada et al. 2000)
SAMP mice (Takeda et al. 1991) (Takeda et al. 1981)	Senescence-accelerated mice, naturally occurring mutant mice (AKR/J background) Accelerated aging Life span: decreased by 27%	Age-associated early decline in immune function Early onset of regression in Ab production to T-independent Ag (DNP-Ficoll) and NK-cell activity Profound defect in Ab response to T-dependent Ag (sRBC), due to the impaired T-helper-cell activity for Ab response Early decline of stimulatory activity in DCs and B-cells Earlier production of autoantibodies against DNA and collagen Type II Some cellular immuneresponses, such as mix lymphocyte reaction, CTL response and DTH reaction are normal	(Hosono et al. 1997) (Hosokawa et al. 1987a) (Hosokawa et al. 1987b) (Yoshioka et al. 1993) (Haruna et al. 1995)

Table 1 (continued)

Strain name (reference)	Molecular description	Immune system	References (immune system)
TERC$^{-/-}$(mTR$^{-/-}$) (Blasco et al. 1997)	Target knockout of telomerase RNA template (mTR) Later generation TERC$^{-/-}$ mice have shorter life span and some phenotypes of accelerated aging	Normal initial development of lymphocytes and other hematopoietic lineages, impaired ability to regenerate hematopoietic populations after 5-FU treatment Reduced proliferative response of T- and B-cells in vitro Decreased T-cell dependent humoral response in vivo, including decreased Ab response and germinal center formation	(Herrera et al. 2000) (Lee et al. 1998)
P53$^{-/-}$mice (Donehower et al. 1992)	Target knockout of P53 gene Tumor suppressor, induces DNA repair, cell cycle arrest and apoptosis Increased incidence of early death, mostly not due to tumor	Thymocyte development initially normal, but develop thymic lymphoma Thymocytes and B-cells are resistant to DNA damage induced apoptosis Accelerated aging of immune system Accelerated age-related accumulation of memory like CD4 T-cells Increased cytokine production and decreased proliferation of CD4 T-cells upon activation in adult mice	(Donehower et al. 1992) (Clarke et al. 1993) (Lowe et al. 1993) (Ohkusu-Tsukada et al. 1999)
P53$^{+/m}$mice (Tyner et al. 2002)	Transgenic mice expressing N-terminus truncated P53 protein Early onset of various aging phenotypes. Effects dependent on wild-type P53 Life span decreased by 17–19%	Significantly enhanced age-associated lymphoid atrophy	(Tyner et al. 2002)

Table 1 (continued)

Strain name (reference)	Molecular description	Immune system	References (immune system)
pL53 mice (Tyner et al. 2002)	Transgenic mice expressing temperature sensitive mutant P53 protein Early onset of aging phenotypes Life span: not documented		
P44-Tg mice (Maier et al. 2004)	Transgenic mice expressing P44, the shorter isoform of P53 Early onset of aging phenotypes Life span: decreased by 40–50%		
Wip1$^{-/-}$mice (Choi et al. 2002)	Knockout of Wip1 (wild-type P53-induced phosphatase1) Serine/threonine phosphatase induced in a P53-dependent manner by DNA damaging agents. Inhibits P53 activity by inhibiting P38 phosphorylation Life span: dramatically decreased in males	Enhanced susceptibility to pathogens Increased inflammation and skin ulcerations Abnormal lymphoid histopathology Decreased T-cell and B-cell proliferative response to mitogenic stimuli	(Choi et al. 2002)
Brca-1$^{-/-}$P53$^{+/-}$ (Cao et al. 2003)	Targeted knockout of Brca-1 gene plus heterozygote knockout of P53 gene. Protein that has been implicated in many normal cellular functions such as DNA repair, transcriptional regulation, cell-cycle checkpoint control Brca-1$^{-/-}$ mice are embryonic lethal, Brca-1$^{-/-}$ P53$^{+/-}$ mice are viable but show dramatic aging phenotypes and significantly shorter life span compared to P53$^{+/-}$ mice		

Table 1 (continued)

Strain name (reference)	Molecular description	Immune system	References (immune system)
Atm$^{-/-}$mice (Barlow et al. 1996)	Target knockout of Atm (ataxia-telangiectasia mutated) Protein kinase that coordinates DNA damage monitoring and repair pathways. Phosphorylates and activates effectors that mediate cell-cycle checkpoint responses Life span: death before 4.5 month due to thymic lymphomas	Decreased generation and survival of DP thymocytes undergoing TCRα gene recombination Severely reduced number of mature CD4+ and CD8+ single positive thymocytes Develop aggressive thymic lymphoma, causing death before 4.5 months	(Barlow et al. 1996)(Matei et al. 2007)(Matei et al. 2006)
Ku86$^{-/-}$mice (Vogel et al., 1999) (Li et al., 2007)	Target knockout of Ku86 Important component of DNA-dependent protein kinase (DNA-PK) that's required for repairing DNA double-strand break (DSB) by nonhomologous end-joining (NHEJ) Early onset of senescence in multiple tissues and organs Life span: decreased by 61–66%	Severe immune deficiency due to early arrest of T- and B-cell development, resulting from failure in VDJ recombination Early onset of age-associated acute and chronic immune reactions in multiple organs, sometimes resulting in sepsis	(Zhu et al. 1996)(Nussenzweig et al. 1996)
Ku70$^{-/-}$mice (Li et al. 2007)	Target knockout of Ku70 Important component of DNA-dependent protein kinase (DNA-PK) that's required for repairing DNA double-strand break (DSB) by nonhomologous end-joining (NHEJ) Early onset of senescence in multiple tissues and organs Life span: decreased by 66%	Deficiency of mature B-cells or serum immunoglobulin Severely decreased thymocytes and peripheral T-cells due to severe impairment in VDJ recombination Significant incidence of $CD4^+CD8^+$ Significant incidence of $CD4^+CD8^+$ thymic lymphoma and disseminated T-cell lymphomas at a mean age of 6 months	(Gu et al. 1997)(Li et al. 1998)

Table 1 (continued)

Strain name (reference)	Molecular description	Immune system	References (immune system)
TTD mice (de Boer et al., 2002)	Target mutation of XPD gene DNA helicase involved in DNA repair and transcription Exhibit many premature aging symptoms Life span: decreased by >50%	Lower NK-cell activity reported in TTD patients	(Mariani et al. 1992)
GH-Tg mice (Palmiter et al. 1992) (Selden et al. 1989) (McGrane et al. 1988) (Bartke 2003)	Transgenic mice expressing growth hormone (GH) under mouse metallothionein I (MT) promoter or rat PEPCK (phosphoenolpyruvate carboxykinase) promoter Transgenic GH is expressed in multiple tissues, such as liver and kidney Exhibit multiple premature aging symptoms Life span: significantly decreased	Increased numbers of migrating cells in laminin-coated transwells Increased CXC chemokine ligand 12 (CXCL12)-driven migration Increased recent thymic emigrants in lymph nodes	(Smaniotto et al. 2005)
Peroxiredoxin$^{-/-}$ mice (Neumann et al. 2003)	Target knockout of peroxiredoxin Small antioxidant proteins, scavenge peroxide and are involved in cellular response to reactive oxygen species (ROS) Life span: significantly decreased	Decreased NK cell cytolytic activity, decreased frequency of NK-cells expressing activation receptor Ly49D Significantly reduced NK-enhancing activity in RBCs from KO mice Development of B- and T-cell lymphomas	(Neumann et al. 2003)

premature aging phenotypes and have drastically decreased life span (Bartke 2003; McGrane et al. 1988; Palmiter et al. 1992; Selden et al. 1989). The development and aging of the immune system in these mice remain poorly studied and await more detailed examination.

3 Mouse Models Demonstrating an Extended Life Span

A. Caloric Restriction Models. Caloric restriction (CR) (limiting food intake without causing nutritional deficiencies) has been the most potent environmental factor that results in consistent extension of life span. The mechanisms responsible for the effect of CR include reducing oxidative damage, lowering GH/IGF level and triggering an innate beneficial response to low-level stressors (Masoro 1996; Merry 2000; Quarrie and Riabowol 2004; Sohal and Weindruch 1996; Weindruch and Walford 1982). CR is also the most extensively studied factor that leads to potent and consistent delay or prevention of immune senescence processes. CR results in delay or reversal of age-related reduction in naïve T-cells, decline of T-cell proliferative response to mitogens and decline in anti-viral immune response, increase in occurrence of tumor and autoimmune diseases and increase in production of inflammatory cytokines (Chen et al. 1998; Effros et al. 1991; Hobbs et al. 1993; Hursting et al. 2003; Spaulding et al. 1997a; Spaulding et al. 1997b; Walford et al. 1973; Weindruch and Walford 1982). Together, these data suggest that overall the immune system benefits from CR. However, in vivo immune responses to pathogens have not been studied to determine if infected CR mice fare better than age-matched mice on ad libitum diets. These studies will provide critical support to the hypothesis that CR benefits immune function.

B. Hormonal Models. A large group of mouse models that have extended life span involve reduced pituitary GH/IGF-1 function (Quarrie and Riabowol 2004). Among these models, the Ames dwarf and Snell dwarf mice have mutate Prop1 gene and Pit1 gene, respectively, both of which result in lowered GH/IGF-1 levels that account for both an extended life span and dwarfism (Bartke et al. 2001; Brown-Borg et al. 1996; Flurkey et al. 2001). Little mice have mutation in Ghrhr gene that encodes GH-releasing hormone receptor and the Laron mice have targeted mutation in growth hormone receptor (GHR) and GH binding protein. The p66shc$^{-/-}$ mice lack the downstream effector of GH/IGF-1 signaling and the IGF-1R$^{-/-}$ mice lack the receptor for IGF-1 (Coschigano et al. 2000; Flurkey et al. 2001; Holzenberger et al. 2003; Lupu et al. 2001; Migliaccio et al. 1999; Zhou et al. 1997). All of these mice have demonstrated extended life spans presumably due to their defective GH/IGF-1 axis. The studies on immune function in these animals are much less extensive than that done with CR mice and many results remain controversial. Among them, the immune senescence of Snell dwarf mice has been the more thoroughly investigated and results show that multiple immune senescence processes are delayed or reversed in these mice (Flurkey et al. 2001; Taub and Longo 2005). These include

Table 2 Mouse Models Demonstrating a Longer Life Span

Strain name	Molecular description	Immune system	References
Klotho-Tg mice (Kurosu et al., 2005)	Transgenic mice expressing Klotho gene under the control of human elongation factor 1α Life span: increased by 20–30% in male, and around 19% in female		
CR mice(Masoro 1996) (Merry 2000) (Sohal, Weindruch 1996) (Weindruch, Walford 1982)	Mice with caloric restriction (limiting food intake without causing nutritional deficiencies) Life span: significantly increased	Delayed or reversed immune senescence, which include age-associated decrease in naïve CD4+ and CD8+ T-cells, decline in T-cell proliferation to mitogenic stimuli, increase in incidence of tumor and autoimmune disease, decrease in anti-viral immunity, increase in production of inflammatory cytokines	(Chen et al. 1998) (Walford et al. 1973) (Weindruch and Walford 1982) (Hursting et al. 2003) (Effros et al. 1991) (Hobbs et al. 1993) (Spaulding et al. 1997a) (Spaulding et al. 1997b)
Ames Mice (Brown-Borg et al. 1996) (Bartke et al. 2001)	Mutation of Prop1 gene Transcription factor in embryonic development of anterior pituitary Life span: increased by 40–50%	Decreased thymocyte numbers	(Duquesnoy 1972)
Snell mice (Brown-Borg et al. 1996)	Mutation of Pit1 gene Pituitary-specific transcription factor Life span: increased by 40–50%	Delayed or reversed immune senescence The age related increase in splenic memory CD4+ T-cells and memory CD8+ T-cells is almost completely prevented, so is the increase in CD4+ and CD8+ T-cells that express cell surface P-glycoprotein The age related decrease in CD4+ and CD8+ T-cell function reflected by IL-2 production and generation of cytotoxic effectors is also near completely prevented	(Flurkey et al. 2001)

Table 2 (continued)

Strain name	Molecular description	Immune system	References
Little mice (Flurkey et al. 2001) (Lupu et al. 2001)	Recessive mutation of Ghrhr (GH-releasing hormone receptor) Life span: increased by 23–25% when maintained on low fat diet		
Laron Mice (Zhou et al. 1997) (Coschigano et al. 2000)	Target knockout of GHR (growth hormone receptor) and GHBP (GH binding protein) Life span: increased by 37–55%		
p66shc$^{-/-}$mice (Migliaccio et al. 1999)	Target knockout of P66shc Downstream effector of IGF-1R Life span: increased by 30%	T-cells are less susceptible to apoptogenic stimuli, and have enhanced proliferation in response to TCR stimulation in vitro	(Pacini et al. 2004)
IGF-1R$^{+/-}$mice (Holzenberger et al. 2003)	Heterozygous for the IGF-1R gene (IGF-1R$^{-/-}$ is lethal) Life span: increased by 26% overall, 33% in female, 16% in male mice and not statistically significant	Decreased T-cell-independent B-cell response by in vivo assay	(Kelley et al. 1998)
TRX-Tg mice (Mitsui et al. 2002)	Transgenic mice expressing thioredoxin driven by human β-actin promoter Small thiol-mediated redox-active protein Enhanced resistance to a variety of oxidative stresses Life span: increased by 35%	Tg mice sustain the Th1 skewed status of Th1/Th2 balance during aging, in contrast to the gradual polarization to Th2 in WT mice Tg macrophages retain the phenotype of reductive macrophages with the ability to produce IL-12, and produce increased amount of NO and reduced amount of IL-6 and IL-10 during aging	(Murata et al. 2002)
FIR-KO mice (Bluher et al. 2003)	Conditional knockout of insulin receptor (IR) in adipose tissues Life span: increased by 18%		
uPA-Tg mice (Miskin, Masos 1997)	Transgenic mice expressing uPA (urokinase-type plasminogen activator) in the brain Reduced food consumption, body size and weight Life span: increased by 16%		

an age-related increase in memory T-cells and a decrease in IL-2 production and generation of cytotoxic CD8+ T-effector-cells (Flurkey et al. 2001).

4 Mouse Models Demonstrating a Normal or Undocumented Life Spans

A. Wnt-β-catenin-TCF signaling models. Wnt-β-catenin-T Cell Factor (TCF) signalling pathway has been shown to regulate thymic involution. TCF-$1^{-/-}$ mice have decreased thymocyte cellularity as early as during embryonic development, resulting from impairment at early stages of thymocyte development (Verbeek et al. 1995). In these mice, thymic involution becomes increasingly severe with age such that by 6 months of T-cells are essentially depleted (Schilham et al. 1998). Mice expressing stabilized form of β-catenin, a partner of TCF-1 for activating target gene transcription, also show enhanced thymic involution with decreased number of all thymocyte subpopulations (Xu and Sen 2003). Thus, increased Wnt signalling, as seen in transgenic mice expressing β-catenin, or decreased Wnt signalling documented in TCF-1-deficienct mice both promote thymic involution. While the precise molecular mechanisms involved in these processes remains under investigation, a balanced Wnt-β-catenin-TCF signalling pathway appears to be essential to maintain thymic function.

B. Ghrelin Infusion and Knockout Mouse Models. Recent studies have demonstrated important roles of Ghrelin in promoting thymopoiesis during aging (Dixit et al. 2007). Ghrelin is a peptide hormone mainly produced by enteroendocrine cells in the stomach in response to negative energy balance. Ghrelin binds to the GH secretagogue receptor (GHS-R) and stimulates growth hormone (GH) secretion from the pituitary. Both ghrelin and GHS-R are expressed by resting and activated human T-cells and exert anti-inflammatory effects on immune cells and systemically in mice (Dixit et al. 2004; Dixit and Taub 2005). Ghrelin and GHS-R expression declines within the thymus with age (Dixit et al. 2007). Mice deficient for ghrelin or GHS-R demonstrate enhanced age-associated thymic involution and decreased numbers of lymphoid progenitor cells in the bone marrow and thymus. Conversely, infusion of ghrelin into old mice significantly improves age-associated changes in thymic cellularity, the number of ETP, CLP, LSK and RTE and improves the TCR diversity of peripheral T-cells (Dixit et al. 2007). Thus, ghrelin emerges as an important factor to promote thymopoiesis during aging.

C. Cytokine Models. Cytokines, particularly IL-7, have been found to be associated with preventing age related thymic involution. IL-7, produced mainly by thymic and bone marrow stromal cells, is a critical trophic factor for both T- and B-cell progenitors, and deficiency of IL-7, its receptors (IL-7Rα and γ_c) and its downstream signalling molecule Jak3 all lead to severe defects in early T- and B-cell development and thus thymic involution and lymphopenia. IL-7 mRNA levels in the thymus decreases 15-fold by 22 months of age (Alves et al. 1995; Andrew and Aspinall 2002; Ortman et al. 2002). Effect of IL-7 administration on

Table 3 Mouse Models Demonstrating Normal or Undocumented Life Span

Strain name	Molecular description	Immune system	References
TCF$^{-/-}$ mice	Target knockout of TCF-1 Transcription factor downstream of Wnt signalling pathway, binds DNA and forms a complex with β-catenin which activates gene transcription	Dramatically decreased thymic cellularity starting from embryonic stage and throughout life; incomplete block at DN1, DN2 and ISP stage of thymocyte development in young mice and complete block at DN1 stage in older mice	(Verbeek et al. 1995) (Schilham et al. 1998)
CAT-Tg mice	Transgenic mice expression stabilized β-catenin under proximal Lck promoter Mediator of Wnt signalling pathway, pairs with TCF-1/LEF-1 to activate gene transcription	Enhanced thymic involution Decreased number of all the thymic subpopulations No drastic reduction in splenic T-cells	(Xu and Sen 2003)
Ghrelin$^{-/-}$mice	Target knockout of Ghrelin Peptide hormone mainly produced by stomach Also expressed by thymocytes and T-cells	Enhanced age-associated thymic involution with reduced thymopoiesis Infusion of ghrelin into old mice significantly improves age-associated changes in thymic cellularity, number of ETP, CLP, LSK and RTE and improves the TCR diversity of peripheral T-cells	(Dixit et al. 2004; Dixit and Taub 2005; Dixit et al. 2007)
GHS-R$^{-/-}$mice	Target knockout of GHS-R (growth hormone secretagogue receptor) G-protein coupled receptor, receptor for Ghrelin	Enhanced age-associated thymic involution with reduced thymopoiesis and diminution of bone marrow and peripheral LSK hematopoietic stem cell population	(Dixit et al. 2004) (Dixit and Taub 2005) (Dixit et al. 2007)
Lurcher mice	LC mutation, autosomal semidominant mutation of glutamate receptor (GluRδ2) gene Selectively expressed on Purkinje cells and LC mutation causes their apoptosis by a high glutamate concentration LC/LC mice die early after birth	LC/+ mice show significant reduction of DP thymocytes and thymic involution by 3 month of age due to enhanced cell death, while peripheral T-cell number is normal compared to control mice	(Mandakova et al. 2005) (Mandakova et al. 2003) (Mandakova et al. 2003)

Table 3 (continued)

Strain name	Molecular description	Immune system	References
Dnmt1$^{+/}$mice	Target knockout of Dnmt1 (DNA methyltransferase 1) Enzyme responsible for maintaining DNA methylation through mitosis	Delayed immune senescence, reflected by significantly delayed age-associated increase in memory-like CD4 T-cells, decline of CD4 T-cell proliferative response and IL-2 production Delayed development of autoimmunity	(Yung et al. 2001)
IL-7$^{-/-}$	Target knockout of IL-7	Severe T- and B-cell lymphopenia resulting from severe impairment of early T- and B-cell development	(von Freeden-Jeffry et al. 1995) (Moore et al. 1996)
IL-7Rα$^{-/-}$ γc$^{-/}$mice	Target knockout of IL-7 receptor alpha (α) chain and the common gamma (γ) chain	Severe T- and B-cell lymphopenia resulting from severe impairment of early T- and B-cell development	(Peschon et al. 1994) (Akashi et al. 1998) (Cao et al. 1995)
Jak3$^{-/}$mice	Target knockout of Jak3 Jak family Tyrosine kinase mediating IL-7 receptor signal	Severe T- and B-cell lymphopenia resulting from severe impairment of early T- and B-cell development Severe defects in NK-cell development	(Nosaka et al. 1995) (Thomis et al. 1995) (Baird et al. 2000)
Lck-IL-7-Tg mice	Transgenic mice expressing IL-7 under proximal lck promoter	Low level IL-7 expression: increased thymocyte number, increased DP and SP thymocyte number Medium level IL-7 expression: no significant changes in thymocyte number High level IL-7 expression: decreased thymocyte number due to impaired proliferation of DN thymocytes	(El Kassar et al. 2004)
IL-7Rα-Tg mice	Transgenic mice expressing IL-7Rα under human CD2 promoter	Normal thymocyte number at birth, rapid decline in thymocyte number with age	(Munitic et al. 2004)

Table 3 (continued)

Strain name	Molecular description	Immune system	References
IL-12β$^{-/-}$mice	Target knockout of IL-12β IL-12 p40 subunit is produced by APCs and regulates function of T-cells and NK-cells.	Accelerated thymic involution due to increased thymocyte apoptosis Accelerated degeneration of thymic structures with decreased cortex/medulla ratio No defect in thymocyte development in young mice No age-associated decrease in IL-12 level produced by APCs in normal mice	(Hsu et al. 2005; Li et al. 2004)
CD11c-Bcl-2-Tg mice	Transgenic mice expressing human BCL-2 under CD11c promoter, in DC cells	Increased frequencies and numbers of DCs, increased turnover/survival (longevity) of DCs Enhanced humoral and cellular immune response in vivo, including enhanced IgG production, T-cell proliferation and cytotoxic activity	(Nopora and Brocker 2002)
CD2-Fas-Tg mice	Transgenic mice expressing Fas under CD2 promoter, in T-cells. Surface molecule that is critical for apoptosis and stimulation during T-cell development	Prevent age-associated decrease in ligand-induced apoptosis and proliferation as well as changes in IL-2, IL-10 and IFNγ production in T-cells; decrease in density of cell surface lipid raft elements possibly influencing cell death or activation	(Zhou et al. 1995)

thymopoiesis and thymic output has been controversial because some reports did not demonstrate significant effects (Fry and Mackall 2002), while others showed that IL-7 increases recent thymic emigrants in periphery without enhanced thymic function and restores immunity in athymic T-cell-depleted hosts (Chu et al. 2004; Fry et al. 2001; Mackall et al. 2001). IL-12, produced mainly by dendritic cells, is another factor that's required for preventing thymic involution during aging process. Aged IL-12$\beta^{-/-}$ mice, but not young IL-12$\beta^{-/-}$ mice, demonstrate accelerated thymic involution compared to age-matched wild type mice. IL-12 enhances the proliferation of thymocytes from aged IL-12$\beta^{-/-}$ and wild type mice in response to IL-2 and IL-7. Thus, IL-12 enhances IL-7 and IL-2 signalling in thymocytes from aged mice and thus may compensate for the age-associated reduction in IL-7 and IL-2 expression and signal, and thereby inhibiting thymic involution in older mice (Hsu et al. 2005). Thus, cytokines can enhance T-cell generation as well as regulate thymic function and peripheral T-cell activity. Additional work is required to define challenges in administrating cytokines to therapeutically enhance T-cell output in older humans.

5 Conclusions

The selection of an animal model for aging or immunosenescence research is dependent on the specific cells, pathways and interventions being considered or studies by an investigator and how such models may physiologically relate to normative aging and immune function. The loss or gain of immune function in a transgenic, knockout or mutant mouse does not necessarily reflect the physiological role of the manipulated molecules within a normal immune response. However, such manipulation does permit one to examine the impact of a pathway or system on life span, aging, immunity and immune development and interactions between various organ systems. Information from these animals can then lead to further examination under physiological conditions and eventually to the development of strategies to manipulate these same systems for possible therapeutic benefit. To date, only a few systems have been examined in the context of aging and much more work is needed. Many of the model systems discussed in this chapter have provided valuable new information on both aging and age-associated immunosenescence, which have lead investigators to initiate more detailed studies on specific molecules and signalling systems as well as the development of additional mouse models to further examine the interrelationships and interactions between these various ligand and signalling pathways. Moreover, some of these studies have even lead to the development of clinical trials in human subjects, such as in several hormonal administration trials. With the completion of the human genome project, we can expect the development of many additional mouse models and our need to understand the role of these molecules in the context of aging, age-related pathologies and immunosenescence will be required.

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Insect Models of Immunosenescence

Jeff Leips

Contents

Abstract: For the past few decades invertebrates have been used extensively as models for understanding the general process of senescence (see reviews by Partridge and Gems 2002; Grotewiel et al. 2005; Keller and Jemielity 2006; Houthoofd and Vanfleteren 2007) and since the 1920's as models for understanding the genes, signaling pathways and cellular processes involved in innate immunity (Brey 1998). These two fields of study have begun to merge as invertebrate models, chiefly terrestrial insects, are increasingly being used to understand both the causes and consequences of age-related changes in immunocompetence. Invertebrates are ideally suited for such studies as they generally have short generation times, short life spans and can be raised in large numbers which improves statistical power for detecting the effects of genetic and environmental influences on functional measures of the immune response. In addition, recently completed genome sequences of invertebrates (e.g., *Caenorhabditis elegans*: C. elegans Sequencing Consortium 1998; *Drosophila melanogaster:* Adams et al. 2000; *Anopheles gambiae*: Holt et al. 2002: *Bombyx mori:* Xia et al. 2004) reveal that many of the genes regulating the innate immune response have homologous genes in vertebrates. Molecular genetic studies have also revealed extensive homology between invertebrates and vertebrates in the signaling pathways that are activated to fight infection (Hoffmann and Reichhart 2002). Thus, the use of invertebrate models is likely to contribute a great deal to our

J. Leips (✉)
Department of Biological Sciences
1000 Hilltop Circle
University of Maryland Baltimore County
Baltimore, Maryland, 21250
Fax: 410-455-3875
Tel: 410-455-2238
E-mail: leips@umbc.edu

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understanding of the genetic influences on immunosenescence in a wide range of organisms, including humans.

Keywords: Immune response • survival • bacterial clearance • *Toll* • IMD • JNK • phenoloxidase • phagocytosis • encapsulation • hemocyte • aging

1 Introduction

One complication in translating what we learn about immunosenescence in invertebrates to vertebrate organisms is that invertebrates rely solely on an innate immune response and lack the components of the adaptive immune system found in vertebrates. While there is some evidence to suggest that invertebrates have immunological memory (Sadd and Schmid-Hempel 2006; Pham et al. 2007), this ability is not well understood. Our current understanding suggests that immunological memory in invertebrates results from remodulation of existing cells to enhance their ability to phagocytize previously encountered pathogens. As such, this is fundamentally different from immunological memory that stems from the use of the B- and T-cells of the adaptive component of the immune response. Looked at in another way however, the lack of the adaptive component of the immune response can be considered an advantage because it allows us to examine the effect of age on the innate immune response without the complications of interactions between the adaptive and innate immune systems. Given the similarity among organisms in the many components of the innate immune response, studies of immunosenescence in invertebrates are likely to provide insight into the effect of aging in the innate immune system in all metazoans, including humans.

This chapter begins with a brief review of the immune system of invertebrates, drawn largely from information on the invertebrate with the most extensively studied immune response, *Drosophila melanogaster.* Those wishing to explore this topic further should consult the many excellent reviews on this topic (e.g., Hoffmann et al. 1996; Tzou et al. 2002; Hultmark 2003; Kurz and Ewbank 2003; Brennan and Anderson 2004; Cerenius and Söderhäll 2004; Christophides et al. 2004; Loker et al. 2004; Gravato-Nobre and Hodgkin 2005; Kim and Ausubel 2005; Mylonakis and Aballay 2005; Schmid-Hempel 2005; Evans et al. 2006; Lemaitre and Hoffmann 2007; Royet and Dziarski 2007; Uvell and Engström 2007). The next section summarizes what we have learned about age related changes in the immune system in invertebrates and includes a discussion of the various techniques used to study this phenomenon. The final section outlines some future directions for the use of invertebrates as models of immunosenescence, highlighting both the challenges and the promise that these organisms provide for a more complete understanding of the causes and consequences of age-related deterioration of the innate immune function.

2 Invertebrate Immunity

There are three general components of the innate immune system shared by most invertebrates: i) a wound response which involves proteolytic cascades and melanization to limit the spread of infection and kill pathogens, ii) cellular responses which involve phagocytosis and/or encapsulation of the invading organism, and iii) local and systemic synthesis and secretion of antimicrobial proteins. Age-related deterioration in any of these components is likely to reduce the effectiveness of the immune system.

2.1 Immune Response Activation

The immune response is typically induced following recognition of nonself by the host. In most invertebrates pathogen recognition is accomplished by pattern recognition receptors circulating in the hemolymph or embedded in the cell membranes of hemocytes (Khush and Lemaitre 2000; Hoffmann and Reichhart 2002 Hultmark 2003; Leulier et al. 2003; Royet and Dziarski 2007). These receptors recognize and bind evolutionarily conserved molecular structures that are unique to the surfaces of different types of microorganisms such as the peptidoglycan components of gram-positive and gram-negative bacteria, β-1,3 glycan in fungi, and phosphoglycan of parasites (Kimbrell and Beutler 2001; Janeway and Medzhitov 2002; Hultmark 2003). Invertebrates produce several classes of these pattern recognition receptors such as peptidoglycan receptor proteins (Royet and Dziarski 2007) and C-type lectins (Nicholas et al. 2004; Ao et al. 2007; O'Rourke et al. 2007), many of which have shared homology with vertebrate receptors (Kang et al. 1998; Khush and Lemaitre 2000; Chaput and Boneca 2007; Griffiths et al. 2007). This shared homology suggests that the general strategies for pathogen recognition are evolutionarily conserved.

In most invertebrates studied to date, the binding of recognition receptors to pathogens initiates a series of proteolytic cascades that result in coagulation of blood and in many species the localized production of melanin (Hoffmann and Reichhart 2002; Cerenius and Söderhäll 2004; Theopold et al. 2004). Interestingly, *C. elegans* is an exception in this case as genes regulating the proteolytic pathway leading to melanization in other invertebrates do not appear to have homologues in the worm genome (Ewbank 2002). Binding of these receptors also initiates signaling pathways to produce antimicrobial peptides (discussed below). The melanization reaction requires the activation of phenoloxidase by prophenoloxidase enzymes which typically reside within particular cell types (the names of these cells vary depending on the organism, Lavine and Strand 2002) and are released following cell disruption. Release of prophenoloxidase enzymes initiates a series of reactions leading to production of melanin (Cerenius and Söderhäll 2004). Deposition of melanin at wound sites contributes to wound healing and the toxic reactive compounds produced during melanin formation are thought to act as disinfectants (Bogdan et al. 2000; Nappi and Ottaviani 2000; Nappi et al. 2000; Cerenius and Söderhäll 2004).

2.2 Cellular Response

The cellular immune responses consist of phagocytosis (engulfment of small pathogens by single cells), nodulation (binding of multiple cells to bacterial aggregations), encapsulation (binding of cells to form a capsule surrounding foreign bodies too large to be phagocytized), and participation in clot formation at wound sites (Lavine and Strand 2002). While phagocytic cells appear to be present in most invertebrates, hemocytes of *C. elegans* do not appear to have phagocytic capabilities (Ewbank 2002). In *Drosophila*, three blood cell types are recognized that participate in various aspects of the immune response: plasmatocytes, crystal cells and lamellocytes (Meister 2004). Plasmatocytes are the phagocytic cells, comprising the largest fraction of the hemocytes in larvae (> 95%, Williams 2007) and are the only blood cell type in adults. Crystal cells are smaller cells containing enzymes for initiation of the phenoloxidase cascade. Encapsulation and nodulation of particles too large to be phagocytized appears to be a unique feature of invertebrates and cell types most often involved in these cell aggregates are plasmatocytes and granulocytes (Jiravanichpaisal et al. 2006). In *Drosophila* encapsulation is carried out only in larvae by lamellocytes which are produced by the differentiation of larval plasmatocytes (Evans et al. 2003).

Phagocytosis involves a complex set of cellular changes involving binding of the pathogen, reorganization of the plasma membrane, induction of cytoskeletal changes and processing of the ingested organism by the phagosome. The use of model genetic organisms, primarily *Drosophila*, combined with large scale genomic studies are beginning to reveal the genes that regulate this process (e.g., Wu et al. 2001, Brennan et al. 2007). Several families of receptor proteins have been implicated as important for the first step in this process, the binding of plasmatocytes to microorganisms. Known receptor families include the *Drosophila* homologue of the mammalian CD36 family of scavenger proteins (Philips et al. 2005), genes in the scavenger receptor class C Type-1 family (Rämet et al. 2001), peptidoglycan receptor proteins (Rämet et al. 2002), proteins with EGF-like repeats (*Eater*: Kocks et al. 2005; *Nimrod*: Kurucz et al. 2007), integrins (Moita et al. 2006) and *Dscam*, a protein with an immunoglobulin domain (Watson et al. 2005; Dong et al. 2006). *Dscam* is of particular interest as this gene has four alternatively spliced exons, different combinations of which can result in the production of over 18,000 different transcripts in *Drosophila* (Watson et al. 2005) and over 30,000 transcripts in *Anopheles* (Dong et al. 2006). This potentially allows recognition and discrimination of a wide diversity of pathogens and may even provide a mechanism for immunological memory in invertebrates. Hundreds of genes appear to be required for internalization and processing of microorganisms as has been revealed by genome wide RNAi analysis (Rämet et al. 2002; Philips et al. 2005; Agaisse et al. 2005; Stroschein-Stevenson et al. 2006) and combined proteomic and RNAi analyses (Stuart et al. 2007). A great deal of research remains to be done to understand how this complex genetic network is organized to regulate phagocytosis.

Invertebrates also produce complement type proteins that act as opsonins to enhance phagocytosis. In the mosquito *Anopheles gambiae,* a circulating thiol-ester

protein (aTEP-1) binds to gram-positive and gram-negative bacteria and stimulates phagocytosis by blood cells (Levashina et al. 2001). The *Drosophila* genome encodes at least four TEP-like genes, three of which are up-regulated after immune-challenge, and one of which seems to be up-regulated by the JAK/STAT signaling pathway (Lagueux et al. 2000; De Gregorio et al. 2001; Agaisse and Perrimon 2004). Proteins encoded by the TEP genes have significant similarity with vertebrate complement proteins of the C3/α_2-macroglobulin superfamily (Levashina et al. 2001, Nonaka and Yoshizaki 2004). This lends support to the idea that the general function of these proteins to promote phagocytosis has been conserved during evolution.

2.3 Humoral Response

The third component of the immune response is the humoral response which results in the local and systemic production of antimicrobial peptides (AMPs). In *Drosophila*, the systemic response of AMPs primarily results from two signaling pathways that regulate NF-κB transcription factors in the cells of insect fat bodies (the functional equivalent of the mammalian liver). Comparative genomic studies have shown these pathways to be generally conserved in most insects (Christophides et al. 2002; Evans et al. 2006; Luna et al. 2006; Cheng et al. 2007). In addition, functional genetic studies and genomic sequence comparisons indicate that the genes regulating the intracellular steps in these pathways are remarkably similar to those regulating the innate immune response in mammals (Hoffmann and Reichhart 2002; Minakhina and Steward 2006).

In *Drosophila*, two pathways regulate the production of up to 20 different AMPs, the *Toll* pathway and the immune deficiency (IMD) pathway (Lemaitre and Hoffmann 2007). The *Toll* pathway, which is similar to the mammalian toll-like receptor (TL-R) signaling pathway, is activated through the binding of the growth factor-like cytokine Spätzle to the *Toll* receptor. *Toll* is a transmembrane receptor first identified as a necessary component for dorsal-ventral patterning (Wu and Anderson 1997). Signaling through this pathway results in the translocation of the transcription factors DIF and Dorsal to the nucleus and upregulation of AMPs such as drosomycin and defensin which act directly on fungi and gram-positive bacteria respectively. The IMD pathway, which exhibits similarity to the mammalian tumor necrosis factor receptor (TNF-R) pathway, is thought to be regulated by the binding of a transmembrane peptidoglycan receptor protein (PGRP-LC) to gram-negative bacteria (Gottar et al. 2002; Choe et al. 2005; Tanji and Ip 2005). Signaling through this pathway results in the translocation of the NF-κB transcription factor Relish to the nucleus and subsequent expression of a number of AMPs generally targeting gram-negative bacteria, although some downstream targets of both pathways are effective against gram-negative and gram-positive bacteria. Recent evidence also suggests that signaling through the IMD pathway also activates the Jun N-terminal kinase (JNK) pathway which contributes to the production of AMPs by the fat body (Delaney et al. 2006). Although the *Toll* pathway is triggered primarily by infec-

tion from fungi and gram-positive bacteria (Ligoxygakis et al. 2002; Lemaitre et al. 1996) and the IMD pathway primarily triggered by gram-negative bacteria, it has been known for quite some time that there is cross talk between these pathways and this provides some level of redundancy in the immune response to infection by these organisms (Lemaitre et al. 1997). A recent model developed by Delaney et al. (2006) proposes that this cross talk results in part from the activation of the JNK pathway whereby the downstream transcription factors of the JNK pathway upregulate AMPs normally targeted by the transcription factors of the *Toll* and IMD pathways. *Toll* and IMD have also been shown to act synergistically, jointly contributing to the upregulation of representative target genes of both of these pathways. This synergism appears to result from the fact that the transcription factors of each pathway can bind to different domains of the promotor regions of these target genes leading to higher levels of expression than expected by the additive effect of each pathway when considered alone (Tanji et al. 2007).

While the fat body of most insects is the primary tissue involved in the systemic response to infection, epithelial surfaces of the epidermis, gut, reproductive system and respiratory tract are also responsible for the constitutive and inducible production of antimicrobial agents to limit microbial growth (Brey et al. 1993; Tzou et al. 2000; Ha et al. 2005; Pinheiro and Ellar 2006, Shapira et al. 2006). Interestingly, production of AMPs at wound or infection sites does not rely solely on the activation of NF-κB pathways, but instead on tissue specific transcription factors and local production of reactive oxygen species (Ferrandon et al. 1998; Ryu et al. 2004; Han et al. 2004; Ryu et al. 2006).

Much of the work in invertebrates has focused on understanding the immune response to bacterial and fungal pathogens; however there is a great deal of recent interest in understanding the immune response to viruses. A major mechanism thought to regulate the response to viral infection is by RNA interference (RNAi), an evolutionarily conserved mechanism for silencing the translation of RNA (Meister and Tuschl 2004). Functional genetic analysis in both *Drosophila* and *C. elegans* indicates that this is also a conserved and effective way to fight viral infection (Schott et al. 2005; Wilkins et al. 2005; Cherry and Silverman 2005; Fritz et al. 2006; van Rij et al. 2006). In addition, expression studies of *Drosophila* artificially infected with the Drosophila X Virus suggest that the *Toll* and JAK/STAT pathways are also involved in mediating an immune response to viral infection (Dostert et al. 2005; Zambon et al. 2005).

3 Invertebrates as Models of Immunosenescence

Many different aspects of immunosenescence have been measured in invertebrates including assessment of the age-specific ability to clear and survive infection and measurements of functional changes in the components of the immune response. Age-specific survival measurements have been obtained either by pricking individuals with a septic needle (Burger et al. 2007), microinjecting individuals with a

standard concentration of bacteria (Adamo et al. 2001; Hillyer et al. 2005), or exposing individuals of different ages to a pathogen (Laws et al. 2004). Typically a large number of individuals of each age group are infected and the subsequent number of deaths scored daily to identify differences in mortality rates following infection. Age-specific abilities to clear bacterial infections are carried out by either pricking or microinjecting individuals of different ages with a standard concentration of bacteria, allowing 24–48 hrs for individuals to clear the infection, and then homogenizing or perfusing individuals and plating aliquots of the solution on agar plates (Kim et al. 2001; Hillyer et al. 2005; Lesser et al. 2006). The resulting number of colony forming units on the plate is an estimate of the ability of that individual to clear the infection. Both age-specific survival and clearance assays provide no functional information on the causes of age-specific changes in the immune response. Functional changes in age-specific components of the immune system that have been measured include age-related changes in expression of immune response genes following infection (Hillyer et al. 2005; Zerofsky et al. 2005), age-specific changes in hemocyte counts (Adamo et al. 2001; Doums et al. 2002; Amdam et al. 2004, 2005; Hillyer et al. 2005), age-specific phagocytic ability of hemocytes (Hillyer et al. 2005), age-specific phenoloxidase activity (Adamo et al. 2001), age-specific encapsulation and melanization ability (Doums et al. 2002) and even age-specific changes in the quantity of fat (used as an indicator of the size of the fat body, the major site of lipid storage and the tissue most responsible for secretion of antimicrobial peptides, Doums et al. 2002).

While it is clear from these studies that the ability to survive and clear a bacterial infection declines with age, the data so far indicate that the underlying causes of immunosenescence differs among species. Adamo et al. (2001) studied various indicators of immunosenescence using a population of crickets, *Gryllus texensis*, that had been collected in the wild but maintained for several generations in the laboratory. They measured sex-specific changes in phenoloxidase (PO) activity (based on an in-vitro enzyme assay with cricket hemolymph that measures the rate of conversion of L-dopa to quinone), counts of hemocyte numbers and survival following injection of *Serratia marcescens* at four ages spanning prereproductive maturity to 4 weeks of age. Males and females were similar in the various immune response indicators up to the age at which males began to display sexual behavior. Sexually mature males had lower phenoloxidase activity and higher mortality following infection compared with younger males and same aged females. This was interpreted by the authors as males trading off immunity for reproduction although the functional connection between PO activity and mating behavior is unclear. The relationship between PO activity and survival following infection was weak however, as 2 week old females had higher PO activity compared to earlier and later ages, but exhibited higher mortality following infection compared with prereproductive ages. The authors speculate allocation of energy toward reproduction offsets the increased protection that would have resulted from higher PO activity, producing a trade-off between reproduction and survival following infection. This hypothesis could potentially be tested with age-matched virgins to minimize the survival cost of reproduction. Unlike PO activity, there was no age-specific change in hemocyte

number and no correlation between hemocyte number and the ability individuals to survive infection at any age. Both males and females had a reduced ability to survive infection with increasing age but were also more likely to die from a sham injection of saline with age. This increase in mortality following sham injection suggests that older individuals may have a reduced ability to withstand the stress imposed by the injection and also possibly a reduced ability to repair a wound site.

An interesting contrast is provided by the work of Hillyer et al. (2005) on the mosquito, *Aedes aegypti*. This study examined age-associated changes in a number of traits including changes in hemocyte numbers, the phagocytic ability of cells, the production of antimicrobial peptides, the ability to clear infection and mortality during the first five days following eclosion. They observed age-related reductions in hemocyte number, a reduction in the ability to clear an artificial injection of bacteria, and an increase in mortality rates following infection. They found no change in the production of antimicrobial proteins following infection at different ages and no decline in the phagocytic ability of hemocytes with age. In this case, age-specific decline in clearance ability and the age-specific increase in mortality rates following infection may be largely explained by a decline in hemocyte number with age. As mosquitoes, like most insects, are not known to produce new hemocytes as adults (either by hematopoiesis or by mitosis, Hillyer et al. 2005) the rate of change in immunocompetence with age may largely depend on the total number of blood cells produced during the larval and pupal periods and the age-specific rate of loss of hemocytes as adults. These conclusions are of course based on observation of correlated changes with age and the causal relationship between hemocyte number and age-specific immunocompetence needs to be further tested. One caveat with this study is that the age-associated changes observed may not reflect senescence per se, as this species can live between 2 weeks to over a month in the laboratory. Further assessment of immune function at later ages, perhaps concurrently with other indicators of senescence such as age specific mortality rates will determine if the interrelationship between immunocompetence and hemocyte number is consistent as the organism ages.

3.1 Implications for Social Influences on Immunosenescence

Comparisons of different species of social insects reveal the potential for age-specific hormonal control of immunosenescence. Doums et al. (2002) implanted workers of two different species of bumblebees (*Bombus terrestris* and *Bombus lucorum*) with nylon filaments at two different ages to measure the age-specific ability to encapsulate and melanize a foreign object. Encapsulation and melanization ability declined in both species with age. In an effort to provide a physiological explanation they measured age-specific changes in the size of the fat body and the number of circulating hemocytes in one species, *Bombus terrestris,* and found only a slight reduction in the size of the fat body and no change in hemocyte number with age. Their conclusion was that the age-related decline in encapsulation and melanization

ability could not be attributed to changes in hemocyte number and the size of the fat body. Of course, this leaves open a number of possibilities yet to be explored to explain their results including age-related changes in the phagocytic ability of the hemocytes. Using a different species of hymenoptera, the honey bee *Apis mellifera*, Amdam et al. (2004) found that immunosenescence is cued by a change in social status as hive workers shift to foragers when they reach 18–28 days old (Winston 1987). This change in social status from hive bee to forager leads to higher levels of juvenile hormone (JH), a reduction in vitellogenin production, a decrease in hemocyte numbers and an increase in the number of pycnotic hemocytes which are not phagocytic. As vitellogenin is an important carrier of zinc the increased pycnosis in hemocytes probably results from low zinc availability. Phagocytic ability of the nonpycnotic cells did not change with age. Interestingly, when foragers are forced to revert to hive duties, juvenile hormone titers are reduced leading to an increase in vitellogenin, increased hemocyte numbers and a reduced number of pycnotic cells (Amdam et al. 2005). The source of these new hemocytes is unclear but may result from hematopoiesis, cell division or mobilization of previously sessile cells. Thus, immunosenescence in this aspect of the cellular component of the immune response in honeybees appears to be under social control and so is reversible, at least temporarily. The fact that *Bombus* do not exhibit age-specific changes in hemocyte number while *Apis* does, may reflect the different social biology of these organisms. *Apis* have a very defined schedule of changing tasks and social status as they age, while *Bombus* are much less regimented, performing both hive and foraging duties for their entire life. As such, JH titres in *Bombus* adults may not change in the manner seen in *Apis* and so the resultant change in hemocyte number is not seen in this organism. A comparative study using these species, and indeed other social invertebrates, in which similar immune response traits are examined with age (combined with changes in survival and clearance ability following infection) would elucidate the general importance of social behavior and hormonal influences in regulating immunosenescence.

3.2 Genetic Basis for Immunosenescence

While the studies discussed above are aimed at understanding the cellular and physiological causes of immunosenescence another set of studies has aimed at understanding the potential genetic contributions to age-related changes in immunocompetence. Kim et al. 2001 were the first to document the existence of an age-related decline in the immune response in an invertebrate system. They used *Drosophila melanogaster* to test the effect of age on the ability to clear an infection of *E. coli.* They used a wild type strain and a strain containing a mutation in *xanthine dehydrogenase* (*XDH*), a gene that influences the production of uric acid, a scavenger of reactive oxygen species and so is a candidate gene for aging. Reactive oxygen species and nitric oxide levels were substantially higher in the mutants and mutants had a significantly higher rate of mortality compared to wild

type flies. Interestingly, in the wild type strain they found a dramatic reduction in their ability to clear bacterial infection as the flies aged, with approximately 60% reduction in this ability from 2–80 days of age. Flies with the mutation were extremely limited in their ability to clear infection and did not show any age-related decline. This is may be due to the fact that the immune response of mutant flies had very little scope for an age-specific decline in immune-response as infection levels were over 25 times higher in mutants than in wild type flies at all ages. Their results suggest that generation of high levels of reactive oxygen is deleterious to all physiological systems, including the immune response. It does not imply that *XDH* directly plays a role in regulating the immune-response or immunosenescence in *Drosophila.*

As the term immunosenescence itself implies age-specific changes in immune function, studies of age-related changes in gene expression are more likely to be useful for understanding genetic influences on immunosenescence than studies using mutants. Microarray studies of flies and mammals have identified a number of age-related changes in the expression of immune response genes a consequence of general aging. In fact, genes known to be involved in the immune-response exhibit some of the most dramatic changes in gene expression with age compared to genes involved in other processes such as metabolism, and growth regulation (Pletcher et al. 2002; Seroude et al. 2002; Landis et al. 2004). Of interest is that immune response genes are typically upregulated with increasing age. This likely reflects a higher pathogen load in older individuals (as demonstrated in *Drosophila* by Ren et al. 2007) and not the age-related deterioration in the control of transcription in general. Another possibility is that older individuals are hyperresponsive to pathogens, and as a consequence show higher levels of transcription following infection compared with younger individuals. This last hypothesis was tested in the experiment described below.

Zerofsky et al. 2005 used changes in transcript levels of the antimicrobial peptide diptericin following artificial infection at different ages as an indicator of age-specific immune function. In their study virgin females of different ages were infected by pricking the cuticle with a needle containing a mixture of bacteria (*E. coli* and *Micrococcus luteus*) that were either live or killed. They found that older females had higher background levels of diptericin than younger females before the artificial infection, reflecting the findings of microarray studies on aging flies. They also found that when flies are infected with live bacteria older females had a higher and more sustained level of diptericin transcription than younger flies. The authors interpreted this as an indication that older flies were less able to clear the infection and so continually maintained production of high levels of diptericin. Unfortunately the bacterial load was not measured in old and young flies and so this conclusion awaits confirmation. When infected with killed bacteria, younger females had higher and more sustained production of diptericin than older flies. Older flies upregulated diptericin production during the first six hours following infection, matching the production of the young flies, but then diptericin transcripts gradually declined. These results suggest that older individuals are not hyperresponsive to infection. Combining the results of this study with observation of higher transcript levels of

immune response genes in older flies implies that older flies carry higher pathogen loads and so have elevated transcription of immune response genes.

The nematode *Caenorhabditis elegans* is an emerging invertebrate model for studying evolutionarily conserved responses to infection (Kurz and Ewbank 2003) and recent work has begun to identify some interrelationship between immunocompetence and longevity. The utility of *C. elegans* for understanding the cellular and physiological processes of immunosenescence may be limited by the fact that many components of the innate immune system common in other invertebrates are missing in this species. As discussed above however, there may be a limited number of such features that are generally responsible for immunosenescence across taxa, even within species. However, given the genetic utility of this species, understanding the genetic basis of immunosenescence in this organism may provide key insights not provided by other insect models. It is clear that *C. elegans* experience higher age-specific mortality when exposed to pathogens (Kurz et al. 2003; Laws et al. 2004). While there is some indication of a connection between genes regulating the immune response and longevity in *C. elegans* (Kurz and Tan 2004; Troemel et al. 2006) additional research is necessary to determine if these same genes act to influence immunosenescence.

3.3 Understanding Natural Genetic Variation Underlying Immunosenescence

In a seminal paper Lazzaro et al. (2004) found that natural populations harbor extensive genetically based variation in the ability to clear infection and indentified single nucleotide polymorphisms in candidate genes associated with this variation. This study has opened up an exciting new direction to identify genes that regulate age-specific changes in immunocompetence. Lesser et al. (2006) used a modified assay developed by McKean and Nunney (2001) to demonstrate a genetic basis for age-related changes in immunocompetence. Using twenty five genetically distinct lines derived from a natural population of *Drosophila* they found that only five lines exhibited an age-related decline in clearance ability (measured at 1 and 4 weeks of age) while eleven lines showed an improved ability to clear an infection with age. The clearance ability of the remaining nine were unaffected by age. They also found no genetic correlation in the ability clear the infection between the two ages. This lack of a genetic correlation in immunocompetence across ages suggests that different genes are responsible for producing the phenotypic variation in clearance ability at different ages. Identification of the genes controlling these age-related changes in the ability to clear infection is an important priority.

4 Future Directions

As highlighted above, many of the age-related changes in the components of innate immunity appear to be species-specific. This undoubtedly reflects the evolutionary divergence in the innate immune system among lineages, the different nature of infective agents faced by these organisms in their particular ecological setting, and also differences among species in the strength of selection to maintain immune function with age. As invertebrates have only recently been used as models to explore the causes and consequences of immunosenescence, perhaps more generalities will be revealed as more species are examined. Much could be gained by a systematic study of age-related changes in key components of the immune response in closely related taxa to establish the extent to which changes in particular components of the immune system (e.g., hemocyte count, phagocytic ability) are unique to particular lineages and which might be generally conserved across taxa. Shared features of the innate immune response that show age-specific decline in function among invertebrates are those most likely to be shared across broader taxonomic groups including vertebrates.

An exciting future goal is to use a combination of techniques for assessing age-specific changes in the immune response in invertebrates to gain a more wholistic view of the mechanisms that underlie immunosenescence. These should include assessing the concentration and identity of pathogen recognition proteins, the levels and killing ability of antimicrobial peptides, measurements of hemocyte numbers and age-specific phagocytic ability. Combining these measurements with age-specific abilities to survive and clear infection will provide the key to understanding which components of the immune response change with age and which have functional consequences for the survival and fitness of the organism.

Shirasu-Hiza and Schneider (2007) have also suggested that we pay more attention to physiological changes in other aspects of the host following infection to identify the causes of mortality when it occurs. As they point out, when humans get sick we measure many physiological indicators that reflect their health and which ultimately contribute to the ability of the individual to survive the infection. They rightly suggest that we expand our understanding of the pathology of infection to monitor changes in aspects of organisms that are not necessarily part of the immune response when experiments are done on model organisms. Additional traits that may be influential include changes in feeding or reproductive behaviors, changes in energy allocation to storage or reproduction, and changes in basic metabolic processes like respiration and the rate and quality of waste produced. As these behavioral, life history and metabolic processes normally change with age, understanding the interrelationship between these physiological characteristics and immunocompetence in the aging organism will provide a more complete understanding of age-related changes in the pathology of infections.

Finally, technological advances are likely to greatly facilitate our understanding of the genetic contribution to age related changes in immunosenescence. Whole genome microarray and proteomic studies are likely to reveal a number of genes that exhibit age-related transcriptional changes prior to and following infection. The key to interpreting these data and identification of important genes will be

to directly test the effect of changes in transcript and/or protein expression with functional tests of immunocompetence. This is perhaps where the real advantage of using invertebrate model systems like *Drosophila* lies. During the past decade there have been a number of tools developed to control the expression of candidate genes in an age—and tissue—and even cell type specific manner in both *Drosophila* and *C. elegans* (e.g., Roman et al. 2001; McGuire et al. 2004; Johnson et al. 2005; Dietzl et al. 2007; Qadota et al. 2007). Controlled up- and down-regulation of candidate genes at different ages prior to and following infection, combined with measurements of survival, bacterial clearance efficiency, or the efficacy of particular components of the immune response (e.g., phagocytic ability) will allow direct tests of the importance of candidate gene expression on age-specific immune function.

Genetic mapping techniques such as quantitative trait loci (QTL) mapping have proven useful for identifying genomic regions that contribute to natural variation in age-specific phenotypes (e.g., Leips et al. 2006) and continued development of these techniques should allow us to rapidly identify the actual genes that contribute to age-specific changes in immune response (Lai et al. 2007). In addition, as age-specific transcriptional controls are likely to contribute to age-specific immunocompetence, the continued use and improvement of expression QTL mapping methods (e.g., Alberts et al. 2007; Jia and Zu 2007) holds great promise for identifying the genes that regulate age-specific expression of those genes that directly contribute to immunosenescence.

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Clonal Culture Models of T-cell Senescence

Graham Pawelec, Jürgen Kempf and Anis Larbi

Contents

Abstract: Studying human T-cell senescence is mostly limited to investigations on peripheral blood ex vivo or on cultured cells in vitro. In both cases, single cell analysis is challenging and many age-associated alterations described are the result of changes in the proportions of the ever-increasing numbers of different T-cell subsets rather than changes to the cells per se. One model avoiding this problem utilises monoclonal populations cultured long-term in vitro. Such T-cell clones (TCC) can be maintained without oncogenic transformation by intermittent antigen restimulation in the presence of growth factors. However, TCC possess finite lifespans (which vary greatly from clone to clone). This TCC model can be used to investigate many aspects of the processes of clonal expansion and contraction essential for adaptive immunity, including biomarker discovery at the genomic, proteomic and functional levels, and to test interventions of possible clinical utility. This chapter describes techniques for the production and maintenance of human TCC in vitro, the impact of culture conditions and oxygen levels on lifespan, and the application of genomic and proteomic analyses in this model.

Keywords: Chronic antigenic stress • Immunosenescence • In vitro culture model • Physiological oxygen level • T-cell clones

G. Pawelec (✉) · J. Kempf · A. Larbi
Center for Medical Research (ZMF)
University of Tübingen Medical School, Waldhörnlestr. 22
D-72072 Tübingen, Germany
Fax: ++49 7071 8884679
Tel.: ++49 7071 253211
E-mail: graham.pawelec@uni-tuebingen.de

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1 Introduction

Long-term propagation of human T-cells became possible after the discovery of "T-cell growth factor", enabling single T-cells to be isolated and cultured for extended (Gillis et al. 1978) but not indefinite periods (Effros and Pawelec 1997). All TCC culture systems relied on the presence of "feeder cells" to facilitate T-cell growth via ill-defined mechanisms of cell contact and cytokine secretion. Nowadays, T-cells can be cultured with defined factors such as the common γ-chain cytokines IL 2, IL 7 and IL 15, together with nanoparticles presenting antibodies to stimulatory surface molecules, commonly the T-cell receptor (TCR) CD3 component and the costimulatory receptor CD28, or together with TCR-cognate antigen and antigen presenting cells (APC) or particles. Notwithstanding all these variations, the basic principles remain the same: to propagate T-cells in vitro it is necessary to provide them not only with exogenous growth factors but also to stimulate them intermittently via their cell surface receptors, most usually the TCR. TCC cultured in this way provide a model for reactivity against antigens which cannot be eliminated by the immune system, i.e. certain parasites, viruses and commonly cancer. When confronted with acute infection, adaptive immunity develops effector responses to clear the antigen; thereafter, excess effector cells are purged from the system and memory cells retained for any future challenges. However, antigens from persistent viruses, notably Herpes viruses, as well as immunogenic cancers, are not cleared, but continuously stimulate specific T-cells. The number of different, mostly CMV-specific, T-cell clonal expansions quantified in vivo first increases and then decreases with age; in the very elderly, the number of remaining clones correlates closely with residual survival time (Hadrup et al. 2006). Thus, chronic antigenic stress (mostly CMV antigens in this case) causes clonal exhaustion and attrition, with clinical consequences. We hypothesize that similar phenomena can occur in younger people harbouring different sources of chronic antigen, especially cancer (Pawelec et al. 2006). The process of T-cell clonal expansion and eventual attrition can be modelled in vitro in tissue culture. We can hope to learn how to modulate this process in vivo by improving and studying the model in vitro.

2 T-cell Cloning

As with fibroblasts, early data on T-cells suggested that clonal lifespan in culture was influenced by age of the donor from whom the starting population was obtained. Thus, clones derived from neonates averaged a larger number of population doublings (PD), than those from adults, especially the elderly (McCarron et al. 1987). Our own more extensive results, collected over many years from multiple cloning experiments, do not support this finding. Individual TCC do have very

varied lifespans, but the overall patterns for T-cells of quite different origins and donors of different ages are remarkably similar, as we have previously reported (Pawelec et al. 2002); thus, clonable T-cells from a centenarian or from a young adult behave similarly, implying that the former have not been functionally compromised. This is consistent with the main age-associated alteration in human T-cells being the changed distribution of T-cell subsets, reflected most prominently in the decreased proportion of naïve cells and the increased proportion of memory cells of different subtypes.

The process of clonal attrition is striking in this in vitro model (Table 1). After 20 PD, representing a clone size of 10^6 cells, about half of the clones originally obtained in each experiment have already been lost. By 30 PD, another half of these is lost, so that only one quarter of the originally clonable cells is still present. By 40 PD (which now represents a very large clone size of 10^{12} cells, at least theoretically if no daughter cells ever die at each cell division), although more clones have been lost, 15% of the original starting clonal population does still remain. These results reflect a steady attrition of T-cell diversity at the clonal level, but with retention of something like 5% of the original CD4 repertoire up to 40 PD and with retention of very rare clones for much longer (some at least to 70 PD). Although difficult to establish, similar clonal attrition probably occurs in vivo as well, at least in infectious mononucleosis, perhaps with quite similar distributions of clonal longevities (Maini et al. 1999). Similar considerations concerning cells from other sources predict that any T-cell, if clonable under these conditions, will behave in a very similar way to any other. This is borne out by the finding that T-cells generated in situ from $CD34^+$ hematopoietic progenitors and those from cancer patients do not manifest greater or lesser average and maximum longevities, respectively (Table 1).

Table 1 Longevities of human T-cell clones under standard culture conditions

Origin	%CE	Clones/ Expts	Percentage of clones reaching:			Max. longevity
			20 PD	30 PD	40 PD	
CD3 (young)	47	1355/15	47	24	15	70
CD3 (old)	52	298/7	48	26	16	77
CD3 (cent)	38	52/3	41	23	17	80
CD3 (CML)	49	35/1	60	35	14	51
CD34 (periph)	55	533/6	31	17	6	60
CD34 (cord)	43	94/2	29	15	5	57

CE, cloning efficiency (calculated from percentage of wells positive in cloning plates). Longevity is expressed as a percentage of established clones (ie. those counted as positive in calculating the CE) which survive to 20, 30 or 40 PD. Origins: CD34+, positively-selected hematopoietic stem cells from peripheral or cord blood; CD3+, normal peripheral T cells; young, apparently healthy donors under 30 yr.; old, healthy donors over 85 yr.; cent, centenarians; CML, a middle-aged donor with chronic myelogenous leukemia in chronic phase treated with interferon.

3 Changes in Behavior over the TCC Life Cycle

The 15–35% of TCC surviving for prolonged periods can be followed longitudinally over their finite lifespans regarding surface molecule expression, activation, signal transduction, cytokine secretion, cytotoxicity, and many other parameters. A typical growth curve of such a clone is shown in Fig. 1 (in this case, a CD4+ clone derived from an octogenarian donor). Growth is well-maintained until 40–50 PD, after which it slows, and the clone is lost at around 55 PD. This slow-down and demise is caused by increasing susceptibility to CD4+ TCC to apoptosis caused by activation-induced cell death, rather than changes to cell division rate. Age-associated alterations discovered in this way over the lifespan of the TCC can be used to screen for similar changes ex vivo in order to validate biomarkers of immune ageing, and they can also be used to test interventions in vitro aimed at preventing or reversing deleterious changes.

3.1 Changes of Surface Phenotype and Function

Bearing in mind the constraints of the cloning procedure, "early passage" TCC will have already undergone at least 22 or 23 PD before sufficient cells are available for analysis and further propagation of the clone, but as mentioned above, at this point there is still a good representation of the original repertoire. TCC can be

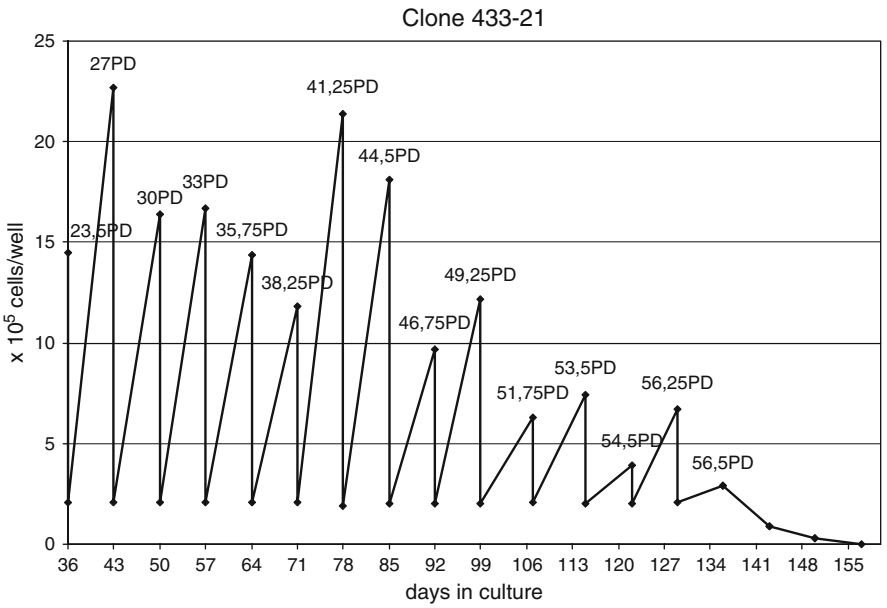

Fig. 1 Growth characteristics of TCC 433-21, showing days in culture plotted against the number of cells per culture well and giving the CPD estimated at each subculture

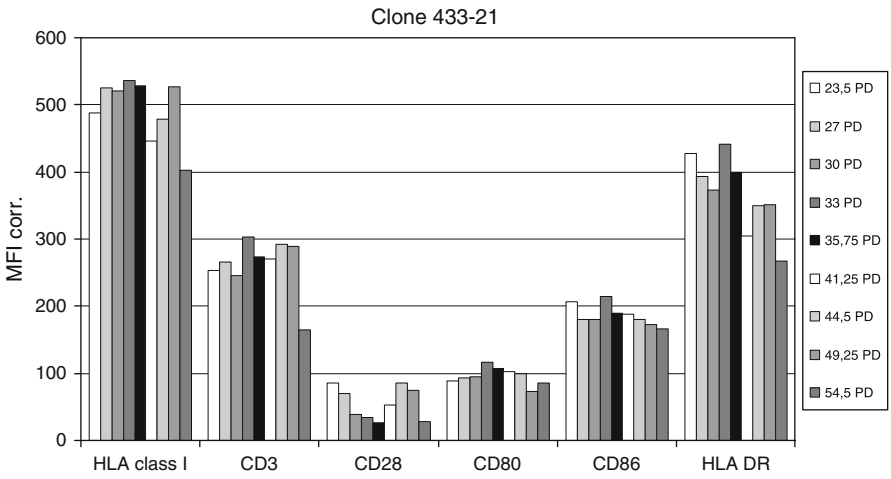

Fig. 2 Surface marker expression of TCC 433-21 giving the CPD estimated at each subculture

analyzed by flow cytometry for changes to expression of an ever-increasing number of monoclonal antibody-defined cell surface molecules, many with important known functions for T-cell responses. A common but not universal age-associated reduction in the level of expression of the costimulatory receptor CD28 has been documented (Pawelec et al. 1997), whereas the level of TCR remains more stable (Fig. 2). This suggests that these cells retain the ability to recognise and respond to antigen but may lack full costimulation, which may contribute to the changes observed in the patterns of cytokines secreted, commonly resulting in decreased levels of IL 2 and increased levels of IL 10 (Pawelec et al. 1997). When comparing the growth curve and the surface marker expression of the same clone, there was a correlation between CD28 expression and the capacity of the clone to grow. CD28 expression is decreasing from 27 to 35 PD and is then re-expressed and finally lost at the end-stage of the clone's lifespan (Fig. 2). This perfectly correlated with the number of cells obtained at each PD (Fig. 1). Nonetheless, other factors certainly play a role in the varied age-associated changes seen in different individual clones. Because T-cell functions are triggered by intracellular signaling via a multitude of surface receptors in addition to the TCR and CD28, any alterations impinging on the membrane (early events) through the cytoplasm (intermediate events) to the nucleus (late events) will influence the final outcome of each encounter with APC for each individual T-cell. This suggests that stochastic events may drive heterogeneity within clonally expanding T-cell populations, a hypothesis for which some evidence does exist.

4 Genomic and Proteomic Analysis

We recently undertook a first global gene expression analysis of early and late passage TCC derived from an octogenarian donor, one of which is shown in Fig. 1. This screening approach allows the hypothesis-free identification of potentially impor-

tant age-associated changes which can be usefully followed up at the protein and functional levels. Array analysis has thus revealed a wide range of differentially expressed genes, including those encoding proteins involved in signal transduction, inflammation, apoptosis, and other processes implicated in senescence (Mazzatti et al. 2007b). Of particular note may be the age–associated upregulation of genes encoding various proinflammatory molecules, considered an important factor in physiological ageing and development of frailty. A similar approach applied at the proteome level may also assist in the discovery of biomarkers of relevance in vivo. Thus, using SELDI-Tof-MS protein profiling, we have identified several protein/peptide peaks which could be associated with T-cell senescence (Mazzatti et al. 2007a). One protein identified through this analysis, profilin-1, hitherto unsuspected in the context of senescence, has important roles in cellular survival, cell division, cytoskeleton remodeling and motility, and may contribute to immunosenescence or possibly cellular senescence in general.

5 Interventions

5.1 Culture Conditions

Interventions that we have tested in the in vitro longitudinal T-cell ageing system described here include culturing TCC in different cytokine cocktails, supplementing with factors such as zinc, attempting to block apoptosis in various ways, and culturing in lower levels of oxygen. The basic culture conditions have remained the same: use of an excess of feeder cells usually consisting of irradiated PBMC pooled from many different healthy donors, culture medium containing human serum or more recently serum free (X-Vivo 15, Lonza, Basel, Switzerland), and a source of growth factors. Briefly, of the many different variants that we have tested, relatively few have had much impact on the growth characteristics and longevities of the TCC. One of these, neutralisation with antibodies of the TNF-α secreted into the culture medium by essentially all TCC, resulted in an increased cumulative PD (CPD) of 10-15 PD (Pawelec et al. 2006). This can translate to a very large number of cells at later passages, and could be used in vivo, since agents that neutralise TNF are licensed for use in humans in diseases such as rheumatoid arthritis. The only other manipulation which has had an impact on TCC longevity, also increasing CPD by 5-15 PD, is to culture the cells in a more physiological level of oxygen, as described in the next section.

5.2 Physiological Oxygen

All of the above-mentioned studies, and indeed most cell culture experiments in any context, are performed at 37°C in humidified incubators gassed with 5% CO_2

but otherwise containing air. This equates to a hyperoxic environment with 20% O_2, not applicable in vivo. We are therefore embarking on experiments in which TCC are cultured in lower oxygen environments. Earlier experiments using 4 different TCC suggested that oxidative DNA damage measured in TCC by a modified Comet assay increased with increasing PD in culture in air. Reducing the oxygen level to 5% led to a marked reduction in accumulated oxidative DNA damage, but contrary to expectations did not lead to an increased longevity of the TCC (Duggan et al. 2004). However, more recent experiments reducing the level of oxygen further to 2% suggest that while some TCC do not show increased longevity under 2% compared to 20% oxygen, the majority does (Fig. 3). Changes in expression of inducible heat shock proteins and other parameters parallel the growth and signal transduction modulations observed in TCC cultured in what we believe approximates a more "physiological" oxygen tension than air. Thus, the interpretation of data derived from the usual type of in vitro culture in air must be treated with caution, and ideally experiments repeated at lower oxygen levels not only in the context of TCC but essentially all other culture-based systems.

5.3 Telomerase Induction

Expanded TCC loss of CD28 expression is associated with reduced telomerase activity and thus telomere length. Transferring the human telomerase reverse tran-

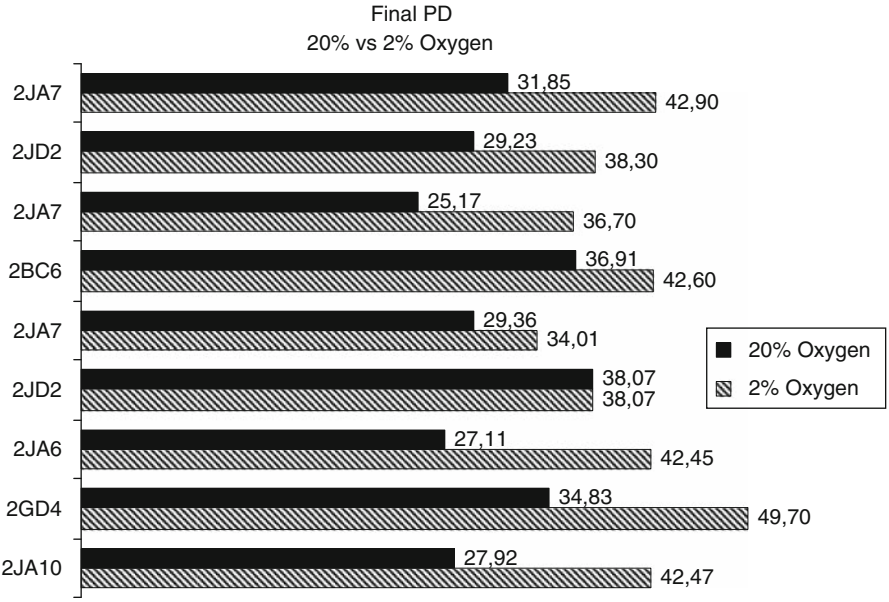

Fig. 3 Longevity of TCC cultured in air or at 2% oxygen. CPD are given for each of 9 individual TCC

scriptase (TERT) gene into T-lymphocytes can increase their lifespan (Rufer et al. 2001). T-cell clones with high levels of telomerase maintained or increased their telomere lengths for extended periods of time. Thus, enforced telomerase expression can increase T-cell longevity.

5.4 Autoantigen-specific T-cell Cloning

The cloning of autoreactive T-cells is more difficult. On early attempts to do so, we concluded that factors other than cytokines including IL 2, IL 4 and IL 7 were required for the expansion of these cells. Mannering et al., recently used CFSE-stained cells, a known cell tracker dye used to assess proliferative capacity of cells, to pre-select responding clones. After stimulation with the auto-antigen acid decarboxylase-65 for 7 days, propidium iodide-negative CD4+CFSE^{dim} cells could be cloned. The cytokine cocktail included IL 2, IL 4 and IL 7 but also IL 15 (5 ng/ml). This resulted in a cloning efficiency averaging from 10 to 15%. IL 15 may therefore be the crucial factor (Mannering et al. 2005).

6 Conclusions

Human T-cell clones can be maintained for extended but finite periods without transformation in tissue culture but eventually cease proliferating at time points up to the Hayflick limit. This remains the case even when known inhibitory factors such as TNF-α are neutralized and when more physiological oxygen tensions are applied, which can increase lifespan but not indefinitely. Only enforced expression of telomerase may greatly extend the lifespan, but this probably also fails to immortalize the cells. The changes which can be investigated longitudinally over the lifespan of the TCC and which reflect in vivo alterations make this a good model for studying human T-cell immunosenescence.

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Mouse Models of Influenza

Ian C. Brett and Bert E. Johansson

Contents

Abstract: Influenza is an enveloped, segmented negative sense RNA virus capable of infecting epithelial cells lining the human respiratory tract. Influenza A and B are important causes of disease in humans. Transmitted via aerosol, the virus possesses two major surface, hemagglutinin (HA) and neuraminidase (NA). HA has binding specificity for sialic acid, and allows viral attachment and entry into the cell. NA cleaves sialic acid residues off glycoproteins or mucoproteins, which aids new progenitor virions in eluting from the cell. The primary method of reducing influenza disease burden has been through vaccination.

Keywords: Influenza • murine • pulmonary titers

1 Introduction

Many different animal models of influenza infection have been used throughout the years, including ferret, mouse, rabbit and swine. However, the mouse is the preferred model for infection because of its ease of breeding, handling and relative low-cost. Importantly the mouse model can be a good predictor of the human response to

I. C. Brett (✉)
State University of New York, Stony Brook School of Medicine, Health Sciences Center, L4, Stony Brook, NY 11794, USA
Fax: +1-631-632-1555
E-mail: ian.brett@hsc.stonybrook.edu

B. E. Johansson
Innovation Sciences, Armonk, NY 10504
E-mail: bertjoh@pol.net

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infection and vaccination. Mouse models have been useful in developing and testing vaccine formulations and pharmacological treatments, as well understanding the pathogenesis of the virus and the dynamics of the host antiviral response. Recent studies have sought to delineate the changes in response to influenza infection or vaccination with age. Studying vaccine efficacy and immune responses in this population is especially important given that the elderly typically show decreased immune response and vaccine efficacy.

This chapter will cover the most widely used mouse strains, discuss gene knockout models, host response to infection and practical aspects of experiments involving mice to provide a starting point for new investigators interested in utilizing mouse models for studying influenza.

2 Inbred Mouse Strains

Investigators requiring mice for research can choose subjects from a large number of inbred and random-bred lines. As part of selecting the ideal murine strain for an experiment, the investigator must choose the genetic state that makes the model a valid representation of the target population. This may include, but is not limited to: selection of histocompatiblity antigens (H2 haplotype), T-helper 1 or 2 skewing of the immune response, or predilections for particular disease states (Table-1). An inbred line consists of a population of great genetic homogeneity, and experiments performed within such a population can test the treatment variability, which in the haphazardly bred mouse might be confounded with genetic variability.

The development of inbred lines of mice in biological research is equivalent to the development of measurement standards and the preparation of reagent and

Table 1 Common Mouse Strains used in Influenza Studies

Strain	Source	H2 Haplotype	Comments
BALB/c	221 (BALB/cJ); 235 (BALB/cByJ) inbred generations	d	Th_2 Skewed; nonaggressive strain; very large reticuloendothelial system
C57BL/6	226 inbred generations	b	Th_1 skewed; High mammary tumor incidence; high mortality in chloroform exposure; low erythrocyte & leukocyte counts; poor LPS response; aggressive strain
Nude (*nu*)	BALB/c-*nu*/+97 inbred generations	d	T-cell deficient, intact B-cell immune system
Nude (*nu*)	C57BL/*lac-nu*+ (B6.Cg-*Foxn1nu*/J)51 congenic generations	b	As above
Swiss Webster	-	outbred	-

Adapted from: Altman PI, Katz DD (1979). Inbred and Genetically Defined Strains of Laboratory Mice; Part 1: Mouse and Rat. Federation of American Societies for Experimental Biology. [1] Source data from Jackson Laboratories Website [22].

analytical grade chemicals and serological reagents. The "reagent grade" animal, the result of carefully controlled inbreeding, permits design and repetition of experiments requiring fine discrimination within an animal species. The inbred mouse line is a population of animals that has attained homozygosis at nearly every locus through the use of a mating system that reduces the number of genetically dissimilar ancestors [44]. Traditionally, the most common practice is rigid brother-sister mating over many generations.

The history of the inbred laboratory mammal is actually the history of the use of the mouse in cancer studies. In order to attain repeatable and controlled systems for studying factors affecting tumor transplantation and for providing insight into why cancer develops, Little and Tyzzer [36] began to breed mice to obtain the necessary genetic homogeneity. Many of the early inbred strains of mice originated from a small number of stocks [12]. This relatively restricted gene pool accounts for the similarities and differences in the classic inbred lines. The history of each inbred mouse line is included in the listing of *Standardized Nomenclature of Inbred Strains of Mice* [54] published regularly. We strongly recommend that the most recent publication of the list of inbred strains be consulted.

The *inbreeding coefficient*, F, is a useful theoretical measurement of the progress of inbreeding, which has retained its usefulness with the experimental use of transgenic animals. It is defined as the probability that both alleles at a locus are identical by descent. It therefore indicates the proportionate decrease of heterozygous loci in the inbred individual relative to those in a representative individual of the starting population. F increases at different rates, depending on the amount of ancestry shared by the mated individuals. F should be used with caution, it is a theoretical value calculated from a pedigree, and not only does it ignore mutational effects, it also ignores effects of selection favoring heterozygotes [12, 53]. Once inbred lines are established, they can be genetically manipulated to establish yet other strains with special attributes for genetic analysis and control. Namely, these are: the *congenic* lines, the *coisogenic* lines, and the *recombinant-inbred* line. A *congenic* line is an inbred line genetically identical to an already established inbred strain except for a short chromosomal segment that bears a distinctive gene of interest. The congenic line is created by crossing the established inbred strain with an individual mouse bearing the distinctive gene of interest; a gene introduced either by breeding or molecular biological techniques. By repeatedly crossing selected carriers of the distinctive gene back to the established inbred strain, in time all introduced genes except the distinctive gene and closely linked genes will have been purged [53]. The locus at which the distinctive gene resides is known as the *differential* locus, the linked genes carried along on the introduced segment are called *passenger* genes, and the original established inbred strain is termed the *partner* or *background* strain. Congenic lines are used (i) to compare effects of genes without the interference of genes in the background, (ii) to easily identify, by the congenic line in which they are carried, individual genes that have similar phenotypic effects, such as histocompatablity genes, and (iii) to assist in linkage studies. In contrast, to the congenic line, a *coisogenic* line is one that differs from its partner strain at a single locus, as a result of a mutation, random or introduced, in an established

inbred line. A *recombinant-inbred* (RI) strain is derived from a cross of two already established highly inbred strains (the progenitor strains), followed by systematic inbreeding as for any other inbred strain. This procedure, with no conscious selection pressure applied, allows the reassortment and fixation of genes from the two progenitor lines [12, 53].

Inbred mice lack the genetic and phenotypic variation seen in outbred mouse populations. The H2 allele is the region that codes for the major histocompatibility complex (MHC), the proteins responsible for processing and presenting processed antigen peptides to T-cells for immune system activation. There are several H2 alleles in circulation in mouse populations, but inbred mice only possess one of these alleles (*e.*g., BALB/c has the $H2^d$ allele). These alleles may vary in the number of different MHC proteins that can be expressed by an organism, or by variation in the protein sequence of amino acids in the cleft that binds and presents processed peptide. However, studies in inbred mice strains infected with *Rickettsia* [2] and murine cytomegalovirus (MCMV) [38] failed to establish a clear role for H2 haplotype in determining susceptibility or resistance to these pathogens. It is likely that several alleles persisted in the population because having the ability to bind a greater number of antigens or having the ability to bind certain antigens conferred some evolutionary advantage to the mouse and allowed persistence of these genes. Therefore, the use of inbred mice homozygous at this locus may skew the results of experiments in an unanticipated manner by artificially restricting the number or type of antigens that can be processed and/or presented by the organism's immune system. Conversely, experimental results may be better than seen in a genetically diverse population. One must be careful in interpreting results and extending generalizations to genetically diverse populations.

3 Specific Inbred Strains

BALB/c is the most common inbred mouse strain used in influenza vaccine studies, developed by HJ Bagg in 1913 (*B*agg *alb*ino). Many substrains of the original BALB/c mouse are now in general use (e.g., BALB/cJ and BALB/cByJ). The substrains share a high degree of genetic identity, but do differ at least one locus [46] and have behavioral and breeding dissimilarities [21]. BALB/c mice have a skewed Th2 immune response; characterized by CD4+ cell expression of IL-4, IL-5, IL-6 and IL-10, cytokines that correspond to activation of the humoral response to infection. IFN-γ and IL-2 correspond to the cell-mediated response. Several studies have demonstrated the BALB/c mouse's skewed Th2 immune response to infection with various pathogens or vaccination [18, 20, 38]. With respect to influenza infection and vaccination, primary infection or primary immunization with different vaccines [20, 16] causes a Th1 immune response in this mouse model, whereas a secondary response to vaccine [16, 55] induces a Th2 type immune response.

The *C57BL/6* mouse is opposite of its BALB/c cousin. This mouse is much more aggressive than the comparatively docile BALB/c. The immune response is skewed toward a Th1 response, which explains the phenotype of resistance to pathogens such as HSV-1, Sendai virus, *Leishmania* and *Rickettsia* [2, 6, 18, 45]. Though this strain has been used to study influenza infection and vaccination, it is usually used experimentally in conjunction with BALB/c mice as a comparison [3].

Nude mice are substrains of BALB/c and C57BL/6 mice homozygous for the *nu* allele carried on chromosome 11, which contains a nucleotide deletion in the *Foxn1* gene. This results in an athymic and hairless animal [19]. The *Foxn1* gene encodes a transcription factor (Forkhead Box) controlling development of the thymic epithelium, important for providing the correct environment for T-cell education [11]. The advantage of an athymic animal model is the ability to study immune responses that require T-cell participation. Sullivan et al. [54] studied infection with A/PR/8/34 (H1N1) found that nude mice had a lower rate of sero-conversion and lower geometric mean titers to virus challenge, indicating that to mount an effective humoral immune response, an intact T-cell immune system is required. However, the mean time to death for nude mice was increased versus BALB/c mice, suggesting that the immunopathology seen in animal models is due in part to Type-1 cell-mediated immunity.

4 Outbred mice

The advantage of using outbred mice for experiments involving infection or vaccine testing is, outbred mice are genetically and phenotypically dissimilar, and therefore a more heterogeneous population. Theoretically, experimental results from studies in such a population may better represent actual results of similar studies in humans. Studies directly comparing influenza infection in outbred models to inbred models are lacking, but studies performed with other pathogens provide data to elucidate differences and similarities of immune responses. Outbred mice have been used for the biological characterization of flavivirus *Alfuy* infection [39], *Coxsackievirus B4 E2* viral spread post-infection [23], Sendai virus infection [45], *Neospora caninum* infection [49] anthrax vaccine studies [14], and a DNA-based FMDV (foot and mouth disease virus) vaccine [4]. In another case [5], outbred mice failed to mount an immune response to vaccination with a plasmid expressing F1 antigen from *Yersinia,* while BALB/c mice demonstrated a robust antibody response. The arsenal of outbred mouse strains to choose from is extensive, however, the Swiss-Webster mouse, its derivative the ICR mouse, are the most used for influenza infection and vaccine studies, though CD-1 and NIH/S mice have been used as well.

Typically, differences between inbred and outbred models were seen in susceptibility or resistance to infection or in response to vaccination, indicating that genetic heterogeneity, likely at multiple loci, is an important consideration for these types of studies. Therefore, based on all studies mentioned in this section, knowledge of the

model chosen for study is essential, and extrapolation of experimental results must be done with extreme caution, and may not be possible in all cases.

5 Transgenic Animals

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to a structural gene, the DNA usually includes other sequences to enable it to be incorporated into the DNA of the host and to be expressed correctly by the cells of the host. Transgenic mice have provided the tools for exploring many biological questions. Two methods of producing transgenic mice are widely used:

1. *The Embryonic Stem Cell Method (Method "1"):* Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, including its gametes.
2. *The Pronucleus Method (Method "2"):* Harvest freshly fertilized eggs before the sperm head has become a pronucleus, then inject the male pronucleus with your DNA. When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo. These embryos are then implanted in a pseudopregnant foster. Every cell in the offspring will contain the gene of interest.

5.1 Random vs. Targeted Gene Insertion

The early vectors used for gene insertion could, and did, place the gene (from one to 200 copies of it) anywhere in the genome. However, if you know some of the DNA sequence flanking a particular gene, it is possible to design vectors that replace that gene. The replacement gene can be one that restores function in a mutant animal or knocks out the function of a particular locus.

If the replacement gene is nonfunctional (a "null" allele), mating of the heterozygous transgenic mice will produce a strain of "knockout mice" homozygous for the nonfunctional gene (both copies of the gene at that locus have been "knocked out").

Knockout mice are valuable tools for discovering the function(s) of genes for which mutant strains were not previously available. Two generalizations have emerged from examining knockout mice:

1. Knockout mice are often surprisingly unaffected by their deficiency. Many genes turn out not to be indispensable. The mouse genome appears to have sufficient redundancy to compensate for a single missing pair of alleles.
2. Most genes are pleiotropic. They are expressed in different tissues in different ways and at different times in development.

6 Experimentally Infecting Mice with Influenza Virus

6.1 Viral Adaptation to Growth in a Mouse

Investigators utilizing murine models to study influenza virus must select or adapt an influenza viral strain suitable to address the experimental question. That is to say, does the murine model system and the infection induced accurately mimic real world phenomenon? In designing such experiments the investigator must address two questions: 1) Does the selected viral strain infect the mouse in a manner that is similar to a human infection in penetrance, viral replication, and replication kinetics? 2) Is the immunity engendered by an experimental vaccine or pharmacologic activity of a drug in the mouse mimic that in the human model? Investigators should be aware that the various immunogenic components of the influenza virus induce very different types of immunity easily reflected in a mouse model system. A more detailed discussion of the immune response to influenza virus can be found in reference [31]. Briefly, antibodies to HA neutralize viral infectivity [26]; antibody to the viral NA [26] and M2 [37] proteins are infection-permissive across a broad range of antibody levels (ie, no reduction in the number of infected subjects) but result in the reduction of pulmonary virus titers below a pathogenic threshold. Antibodies to M1 and NP can be found in the sera of animals immunized with whole virus vaccines, purified protein preparations and after infection. These studies failed to demonstrate a significant role for these antibodies in the amelioration of disease [25]. Despite evidence that live and inactivated influenza vaccines induce cross-reactive T-cells in humans [34] and mice [41], reinfection with homologous or heterotypic virus occurs. The level of anti-influenza CTLs correlates with the rate of viral clearance but not alter susceptibility to infection or subsequent infection [41].

Adaptation of influenza virus by serial passage in new host invariably results in the selection of mutants better equipped to replicate and spread within the new host. Most influenza virus strains grow readily in the mouse lung or murine tissue culture; thus influenza viruses, which are inherently cytolytic, adapt or become more damaging to the animal host not by changes in capacity to infect (which they already must possess) but by mutational changes that permit attainment of higher titers in the host. A common feature of adaptation of both influenza A and B viruses to the mouse has been the emergence of virus characterized by a more rapid growth rate [56, 35] and the capacity to reach higher concentrations in the lung [50]. Adaptation has been studied in the laboratory for many years. Changes in viral phenotype have been noted concomitant with sequential passage of virus in mice [8, 9, 40]. Serial passage of influenza A viruses in the mouse lung has been associated with antigenic changes [15], increased resistance to mucoprotein inhibitor, and changed sensitivity of viral HA to thermal inactivation [31]. Co-variation of these phenotypic changes with attainment of mouse lung virulence has not been unequivocally established. Whether adaptation comprises a complex series of mutational events or primarily entails selection of preexisting viral variants is not clear. Mouse-adapted virus is characterized by both a faster

growth rate and the capacity to attain higher titers in the lung [13]; indicating that both selection of preexisting mouse lung replication mutants and their subsequent mutation occur.

There have been several studies examining the specific mutations and in which viral genes that confer a mouse-adapted phenotype. Adaptation of human influenza virus to mice by serial passage results in the selection of highly virulent variants that have acquired mutations in multiple genes [8, 9, 40]. Analyses of the genetic basis for virulence by using reassortants that possess mixtures of genes from virulent and avirulent strains have identified various groupings of genes, which in aggregate implicate all eight genome segments [9]. Brown et al. [9] demonstrated in H3N2 virus that a group of 11 mutations convert an avirulent virus to a virulent variant. Thirteen of the 14 amino acid substitutions (93%) detected among clonal isolates were likely instrumental in adaptation because of their positive selection, location in functional regions, and or independent occurrence in other virulent influenza viruses. Mutations in virulent variants repeatedly involved nuclear localization signals and sites of protein and RNA interaction, implicating them as novel modulators of virulence. Mouse-adapted variants with the same HA mutations possessed different pH optima of fusion, indicating that other viral genes can modulate HA fusion activity. Experimental adaptation resulted in the selection of three mutations that were in common with the virulent human H5N1 isolate A/HK/156/97 [9]. Similarly, adaptation of the A/FM/1/47 H1N1 strain to mice resulted in selection of a variant with increased virulence. Complete sequence analysis identified mutations in the PB1, PB2, HA, NA, and M1 genes; all five mutations were shown to control virulence but also the replicative capacity in the mouse. The HA, NA and M1 mutations increased yield in all three hosts whereas in combination the PB1 and PB2 mutations were host restrictive changing the virus to a mouse specific strain. However, the HA mutation increased virulence largely independent of increased growth indicating a change in pathological properties [8]. Serial passage of an initially avirulent influenza B virus, B/Memphis/12/97, resulted in the selection of a variant that was lethal in mice. Sequencing data suggested one change in the C-terminal domain of the M1 protein, an asparagine to a serine at position 221, was responsible for acquisition of virulence and lethality [40].

6.2 Practical Experimental Points

Viral strains suitable for adaptation to the mouse can be derived from wild-type [50–52], classic reassortant virus [24, 28–30] or products of reverse genetics [57]. Use of each method has distinct advantages and disadvantages. Use of wild-type strains can be a quick, simple and allows for opportunities to mimic wild-type antigenic exposures from both the internal and surface antigens. However, adapting the strain to mouse may prove laborious, difficult to standardize and low-yield growth in embryonated chick eggs may limit experimental choices. Whereas, use of the classic reassortant and reverse genetic techniques offers the advantage of placing the surface glycoproteins,

HA and NA of a wild-type strain onto a background of internal proteins derived from A/PR/8/34 (H1N1) virus, as done annually, in the production of conventional inactivated influenza vaccine. A/PR/8/34 has an optimal growth temperature of 39°C -the average body temperature of a BALB/c mouse [1], is permissive to growth in a variety of experimentally useful tissue culture cell-lines including Madin-Darby Canine Kidney (MDCK) [31] and there are many commercially available serologic and immunologic reagents compatible with this system. The disadvantage of reassortants and reverse genetics is the need to expand the virus in embryonated chicken eggs to obtain sufficient virus to do the experiment. Passage in chicken eggs leads to deadaptation to the mouse [24, 28– 30]. A reverse genetics plasmid kit containing genes encoding influenza internal proteins from a mouse adapted strain is not available; therefore viral products of reverse genetics will require adaptation passages.

Traditionally, influenza virus has been adapted to increased penetrance or viral growth by sequential passage of virus in mouse lungs [9, 31, 50], with limited passage for expansion in embryonated chicken eggs, which may select for virus less adapted to growth in the mouse lung [24, 50–52]. Passage in MDCK selects for and preserves mouse-adapted characteristics. However, the total amount of virus produced in tissue culture is less than in eggs. Both methods require purification of virions from the growth medium; simple centrifugation and sucrose gradient centrifugation can produce usable high yields of infectious virus [48]. There are commercially available affinity chromatographic methods. Irrespective of the selection method chosen for passing the virus close attention must be paid to dilution (concentration) of virus used in passage studies. Successful adaptation to the mouse lung can be achieved with a wide range of varying viral concentrations and multiplicity of infections (*moi*). Too low of a *moi* may be insufficient for productive infection, to high of a concentration may result in multiplicity interference. Often prior to an experiment the optimal range of viral dilutions is unknown therefore titration of the virus in mice using several dilutions is warranted. In both reassortment and selection of viral strains, one must be cognizant of the enormous mutation rate of influenza (and other RNA viruses) and their apparent requirement for high *moi* for maintaining fitness in a given host system [33, 43]. In other words, either the introduction of populations limited in genetic diversity or the attempt to clone high titer virus by limiting dilution may lead to the establishment of a less fit virus. If such a virus is then maintained by high dilution passage, it will never regain adapted vigor. This phenomenon is known as a genetic "bottleneck" or "Muller's ratchet" [10]. In our laboratory, we have observed the loss of rapid growth and viral titer in mice if passed too early at high dilution. The practical lessons are: pass at sequential low dilutions early; then after sufficient dilutions and passages to escape unwanted nonadapted genes, passage should be maintained at a dilution sufficient to assure a reasonable gene pool. If using A/PR/8/34 reassortants this is in the range of 10^{-4} to 10^{-5} egg infectious dose (EID_{50}). In general, following infection with influenza virus in the mouse peak viral replication is at 3–4 days post inoculation and peak pulmonary lesions are seen on day 7 postinoculation [28–31, 51, 52, 57]. Although, there are viral strains that can peak earlier, it is our opinion that any mortality among the mice prior to day 3 should be examined by assaying the lungs in a tissue culture based plaque assay.

6.3 Experimental Techniques for Infecting Mice with Influenza Virus

Influenza virus has been used to infect mice via several routes of entry including intracerebral inoculation [42, 47], discussion will be limited to the most common techniques: intranasal instillation and aerosol exposure.

Intranasal instillation: Mice should be lightly anesthetized with Metofane anesthesia (Mallinckrodt Veterinarian) or another non-ether, non-chloroform based anesthetic agent. Light anesthesia should induce a state where the animal is not in distress and is mildly hyperpneic. The animal should be firmly held behind its neck and in one hand between the thumb and forefinger, the tail can be held by the investigator's fifth digit. With the animal held supine, 50 µl of live virus or phosphate-buffered saline can be instilled intranasally by pipette or syringe [17, 24, 51, 52]. A range of viral dilutions is recommended.

Aerosol exposure: Shulman and Kilbourne described a technique [24, 50–52] utilizing a retired autoclave. By the use of air pumps the interior of the autoclave could be maintained at a steady negative pressure relative to the room. Dilutions of virus could be aerosolized under positive pressure via an air pump. The aerosolizing devices are delicate, fragile and expensive, which in part contributes to the popularity of intranasal instillation. No mouse-to-mouse transmission has been observed [24, 51, 52].

6.4 Measurement of Endpoints of Infection

When initiating an experimental protocol decisions regarding how endpoint of infection will be measured should be made *a priori.* The options are: measure virus pulmonary titers in plaque assay [32] or by PCR [57] at specific time points; calculation of mouse infectious dose 50 (MID_{50}), monitor mortality/lethality to calculate a lethal dose 50 (LD_{50}) [9, 50]; measure weight loss and recovery [27]. Serologic studies (e.g., antibody titers to HA, NA or other viral antigens) and cellular immunologic studies (e.g., B-cell, T-helper and CTL assays) can be easily included [28–30]. Briefly, these assays are:

1. *Mouse Infectious dose (MID)* is a measure of the amount of infectious virus in a given sample, not the number of virions but a functional assay of infectious capacity. MID_{50} is the calculated dilution of a viral preparation that is expected to infect 50% of the mice inoculated. Usually stated as units of MID_{50} i.e., 1 MID_{50}; 100 MID_{50}. For example if a 10^{-5} dilution infected 50% of the mice exposed then the MID_{50} is 10^{5}; 100 MID_{50} would be a 10^{-3} dilution. The same concept is true for EID_{50} and $TCID_{50}$.
2. *Mortality-lethality* is a measure of how many and when animals die post exposure. A *Lethal dose* (LD) is often used, as with MID, a lethal dose 50 (LD_{50}),

a dilution of virus inducing an infection lethal to 50% of the animals exposed can be calculated. Relative risk (RR) and odds ratios (OR) can be extracted from these data.

3. *Mean pulmonary titer (mPVT)* can be directly measured in hemagglutination assay of lung preparation, or to increase sensitivity, HA assay can performed after lung preparations are inoculated into chicken eggs. Tissue culture assays allow calculation of *viral plaque forming units (PFU)*. In monolayer cell cultures maintained under agar, it can be shown that influenza-virus induced plaques usually are initiated by single virions [31]. Therefore, counting the number of plaques in a given dilution of lung preparation provides an estimation of the pulmonary viral load [7].
4. *Pulmonary lesion/plaques* lungs can be removed from infected animals on day 7 post exposure and the distinct plum colored pulmonary lesions induced by influenza cytopathology can be measured and counted.
5. *Weight gain/loss* is a sensitive measure of illness in the murine model for influenza. Infected animals become less active, eat and drink less in the context of increased metabolic demands of an acute illness. Daily weighing accurately detects weight loss and recovery. Weight loss and lack of recovery are good predictors of mortality [27].

7 Conclusion

The murine model for the study of influenza is well established. Although the mouse is not an exact mimic of avian, equine or human influenza knowledge of the systems experimental strengths and flaws can produce valid and reliable data.

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A Transgenic Dwarf Rat Strain as a Tool for the Study of Immunosenescence in Aging Rats and the Effect of Calorie Restriction

Isao Shimokawa, Masanori Utsuyama, Toshimitsu Komatsu, Haruyoshi Yamaza and Takuya Chiba

Contents

Abstract: Immunosenescence or dysregulation of the immune system may accelerate the aging process and shorten the lifespan in animals. Calorie restriction, a well-known nutritional intervention for longevity in laboratory animals, retards immunosenescence and modulates the immunosystem. These effects of CR could contribute partly to extending the lifespan. A transgenic (Tg) dwarf rat strain, in which the growth hormone (GH) axis is selectively suppressed, lived longer and exhibited several phenotypes similar to those in CR rats, suggesting an important role for the GH axis in the effect of CR. Here, we describe the longevity, pathology, thymic and splenic lymphocyte subpopulations and response to endotoxin in Tg rats in comparison with CR rats. The findings support the importance of the GH axis in the effect of CR on the immune system and, in particular, longevity. The Tg rats could be a useful tool to better understand the molecular mechanisms underlying the antiaging effect of CR.

I. Shimokawa (✉) · T. Komatsu · H. Yamaza · T. Chiba
Department of Investigative Pathology, Unit of Basic Medical Science
Graduate School of Biomedical Sciences, Nagasaki University
12-4 Sakamoto I-chome, Nagasaki City 852-8523, Japan
Tel.: +81-95-819-7051, Fax: +81-95-819-7052
E-mail: shimo@nagasaki-u.ac.jp

M.Utsuyama
Department of Pathology and Immunology
Aging and Developmental Sciences,
Tokyo Medical and Dental University Graduate School

T. Fulop et al. (eds.), *Handbook on Immunosenescence*,
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1 Introduction

Innate and acquired immunity is important for animals not only to protect against infection of microorganisms but also to inhibit a number of diseases including cancers, which are prevalent in aged animals. The immune system is, however, a 'double-edged sword' as the inflammatory hypothesis of aging presumes [1]. Aging-related dysregulation of the immune system could accelerate the aging process and shorten the lifespan of animals by activating proinflammatory processes that lead to excess generation of reactive oxygen and nitrogen species that potentially injure cellular components.

Restriction of food intake with supplying essential nutrients for survival in laboratory animals, referred to as calorie restriction (CR), reduces morbidity and mortality [2]. This effect has been called 'anti-aging' because many laboratories also confirmed retardation or inhibition of the pathophysiological aging processes by CR, an effect first reported by McCay et al [3]. Although proper modulation of the immune system could be one of the main mechanisms by which CR affects aging and longevity in animals, our knowledge is incomplete.

Another line of studies in the biomedical gerontology have found that a single gene, if spontaneously mutated or genetically engineered, could prolong the lifespan of organisms. Although this evidence was initially limited to invertebrates, over 10 genes are now reported in laboratory rodents (Fig. 1). Many of these longevity genes are clustered into the signaling pathway of GH and subsequently insulin-like growth factor (IGF)-1 or insulin. Attenuation of these signaling pathways favors longevity. The GH-IGF-1/insulin pathway is important for understanding the mechanism underlying the effect of CR, because CR is also known to reduce plasma levels of IGF-1 and insulin in laboratory animals [4, 5]. Because GH and IGF-1 are known to modulate the immune system [6], we may hypothesize that CR exhibits the antiaging effect through suppression of the GH-IGF-1/insulin axis and thus modulating immune system.

In this study, we describe some traits of the transgenic dwarf (Tg) rat strain that we established as an aging research model [7, 8], in comparison with CR rats. Published data have indicated that Tg rats fed ad libitum had phenotypes similar to wild-type CR rats for body weight, food intake, fat content, glucose tolerance, insulin sensitivity, and adiopokines such as adiponectin and leptin [8-10]. Although, at present, the immunological findings of Tg rats are limited, this rat model could provide knowledge on the relevance of immunosenecence to aging and longevity. Similarily, data on immunosenescence in other longevity models are limited and, thus, future analyses of the immune system in these models are needed to explore the role for each gene or gene product in immunosenescence and aging.

2 Animal Husbandry and General Data

2.1 Transgenic Dwarf Rats and Husbandy

The Tg rats, with a genetic background of Jcl:Wistar (Japan Clea, Inc., Tokyo, Japan), were produced from founders created by introducing fusion genes into rat embryos [11]. The transgene consisted of four copies of thyroid hormone response

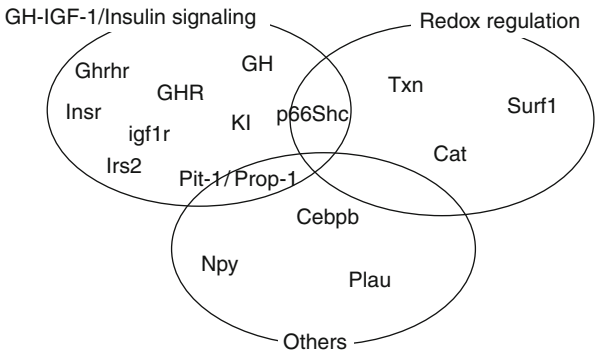

Fig. 1 Longevity genes in rodents. Genes that extend the lifespan of mice or rats, if spontaneously mutated or genetically engineered, are listed. The genes can be classified into three categories; genes that are associated with the GH-IGF-1/ insulin signaling pathway, redox regulation and other genes, although these categories are not mutually exclusive. ***Prop-1*** (spontaneously mutated mice for paired like homeodomain factor 1 gene; [15]): ***Pit-1*** (spontaneously mutated mice for POU domain, class 1, transcription factor 1 gene; [32]: ***p66Shc*** (knockout mice of src homology 2 domain-containing transforming protein C1 gene; [22]: ***Ghrhr*** (spontaneously mutated mice for growth hormone releasing hormone receptor gene; [32]: ***GHR*** (knockout mice for growth hormone (GH) receptor gene; [16]: ***GH*** (over-expression rats for antisense GH gene; [7]: ***igf1r*** (knockdown mice for insulin-like growth factor I receptor gene: [23]: ***Insr*** (mice for adipocyte-specific disruption of the insulin receptor gene; [33]): ***Txn*** (Overexpression mice for human thioredoxin gene: [34]): ***Cat*** (Mitochondria-specific overexpression mice for human catalase gene; [35]): ***Plau*** (brain-specific over-expression mice for urokinase type of plasminogen activator: [36]): ***Npy*** (overexpression rats for neuropeptide Y gene; [37]): ***Cebpb*** (knock-in mice for CCAAT/enhancer binding protein (C/EBP) beta gene; [38]): ***Kl*** (overexpression mice for klotho gene; [39]): Irs2 (whole body or brain-specific knockout mice for insulin receptor substrate 2 gene; [40]): ***Surf1*** (knockout mice for surfeit gene 1; [41])

elements, rat GH promoter, and antisense cDNA sequence for rat GH. The rat GH antisense gene was expressed in the pituitary gland of Tg rats as early as 3 weeks of age. Reverse transcription PCR analyses in Tg rats at 6 months of age confirmed that antisense GH-mRNA was expressed in the pituitary gland, spleen, and thymus, but not in the lungs, liver, heart, kidneys, and testis [7].

F1 hybrid rats were also generated at our laboratory animal center by mating female W rats with male Tg rats to moderate the reduced level of suppression of the GH-IGF-1 axis; the animals were referred to as tg/tg, tg/–, and –/– regarding the presence of the transgene.

At 4 weeks of age, weanling male rats were transferred to a barrier facility, housed separately, and maintained under specific-pathogen-free conditions. The animal husbandry is reported in detail elsewhere [7, 8]. Briefly, rats were provided a standard diet and tap water throughout the experiment (the AL group). The CR regimen in each rat group was initiated at 6 weeks of age. Rats in the CR group were provided 30% less food of the AL group by feeding them with two portions of food every other day 30 min before the lights were turned off.

Table 1 Characteristics of Tg and CR rats at 6 months of age

	(-/-)		(tg/-)		(tg/tg)	
	AL	CR	AL	CR	AL	CR
Body weight (g)	478.9 (34.1)	342.0 (16.9)*	316.6 (32.1)#	226.2 (15.4)*/#	199.1 (8.9)#	124.4 (10.5)*/#
Food intake (g/day)	21.6 (3.5)	15.9	17.0 (2.2)#	11.7	11.2 (1.3)#	7.9
Blood glucose (mg/dl)	126 (34)	112 (12)	106 (18)	90 (16)	106 (17)	n/a
Serum insulin (ng/ml)	102 (49)	15 (10)*	22 (18)#	20 (26)	21 (19)#	n/a
Plasma IGF-1 (ng/ml)	1094 (119)	864 (79) *	627 (90) #	346 (40)*/#	266 (23)#	170 (11)*/#
Plasma GH (ng/ml)	157.3 (55.0)	178.3 (26.7)	172.1 (44.1)	129.6 (35.9)	142.8 (33.9)	126.0 (46.0)
Pituitary GH-mRNA	1.00 (0.25)	0.81 (0.15)	0.34 (0.10)#	n/a	n/a	n/a

Values represent the mean (standard deviation) of 3–6 rats. (–/–); wild type rats. (tg/–); transgenic hemizygotic rats. (tg/tg); transgenic homozygotic rats. AL; ad libitum feeding rats. CR; 30% calorie-restricted rats. * $p < 0.05$ versus (vs) group AL in each rat group. #, $p < 0.05$ vs (–/–) of each diet group.

2.2 Characteristics of Tg and CR Rats at 6-Months of Age

The body weight and food intake in the AL condition decreased comparatively in tg/– and tg/tg rats, gene-dose dependently (Table 1). Following the 30% CR regimen for each AL group, the CR group showed 30~40% reduction in the body weight compared with the respective AL group. It should be noted that both the body weight and food intake in (tg/–)-AL rats were similar to those in (–/–)-CR rats for the first 24 months in the lifespan study [8].

Blood glucose levels under non-fasting conditions were slightly reduced in (tg/–)-AL and (tg/tg)-AL rats, while not significantly different between (tg/–)-AL and (tg/tg)-AL rats. CR also reduced the blood glucose level. The serum insulin level was significantly lower in (tg/–)-AL and (tg/tg)-AL. CR in (–/–) rats led to a significant reduction in the insulin level; there was no additional decrease by CR in (tg/–) and (tg/tg) rats.

The plasma IGF-1 concentration, an index for the degree of suppression of GH-IGF-1 signaling, decreased by 40% in (tg/–)-AL rats and by 75% in (tg/tg)-AL rats compared with (–/–)-AL rats. CR in each rat group further decreased the IGF-1 level; the level in (–/–)-CR rats was reduced to 80% of the level of (–/–)-AL rats. Thus the level was slightly lower in (tg/–)-AL rats than (–/–)-CR rats. The pituitary GH-mRNA level was also reduced by 20 and 66% in (–/–)-CR and (tg/–)-AL rats, respectively.

2.3 Longevity and Pathology

The lifespan at the 25th percentile point was increased by 10 and 11% in (tg/–)-AL and (–/–)-CR rats, as compared to (–/–)-AL rats (Figure 2); however, it was reduced

Table 2 Probable causes of death in Tg and CR rats

	(–/–)		(tg/–)		(tg/tg)	
	AL	CR	AL	CR	AL	CR
Total	30	26	30	30	39	17
Neoplastic (subtotal)	15	18	22	14	37*	11
Leukemia/lymphoma	0	1	2	3	21#	8
Pituitary adenoma	5	8	11	2#	1	0
Others	10	9	9	9	15	3
Non-neoplastic (subtotal)	15	8	8	16	2	6

Data represent the number of rats. The proportion of each category or disease was analyzed by χ^2 test or Fisher's exact test. * $p < 0.05$ versus (–/–)-AL rats. # $p < 0.05$ versus (tg/–)-AL rats.

by 9% in (tg/tg)-AL rats. Postmortem examination indicated that 50% of (tg/tg)-AL rats died of leukemia (Table 2); in contrast, only a few (tg/–) and (–/–) rats suffered from the disease. Furthermore, pituitary adenoma was less frequently in the cause of death in (tg/tg) rats. Our previous analysis indicated that moderate suppression of GH increased lifespan mostly due to the delay or inhibition of non-neoplastic causes; the effect on neoplastic causes was minor [7, 12].

Most of the rats that were considered to die of leukemia showed hepatosplenomegaly. Microscopic analysis and immunohistochemistry with an antibody for NK cells indicated that most of the cases were mononuclear large cell leukemia, which is frequently observed in inbread F344/N rats [13, 14]. Because this type of leukemia is not commonly observed in outbread Wistar rats and that the tg/tg rats were expanded from a pair of founder Tg rats, it is likely that this specific type of leukemia become tangible during the inbreeding process in (tg/tg) rats. Conditional survival analysis was performed to determine if the leukemic death was eliminated (Figure 2). However, the survival did not exceed that in (–/–)-AL rats.

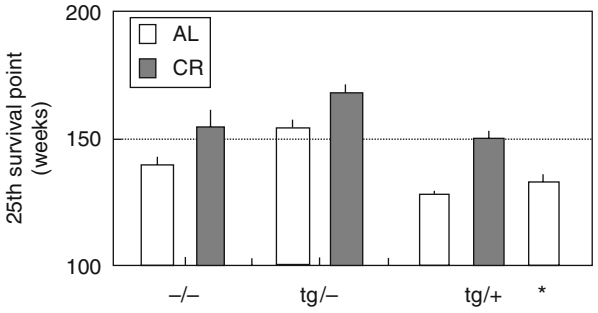

Fig. 2 Lifespan of transgenic dwarf rats: the effect of calorie restriction. Each bar represents the age (weeks) of 25th percentile survival point of lifespan (+ standard error). –/–, wild type rats. tg/–, hemizygotes for the transgene. tg/tg, homozygotes for the transgene. AL, a group of rats fed ad libitum. CR, 30% calorie-restricted rats. N = 30 for each group at the start of the study, with the exception of the (tg/tg)-AL group (N = 55) and (tg/tg)-CR group (N = 35). Survival data is described in more detail elsewhere [7, 8], except for the (tg/tg)-CR group. * Conditional survival if leukemia was excluded from the causes of death, i.e., leukemic death was considered to be the same censorship as random sacrifice of rats. The details of the procedure for the conditional survival are described elsewhere [42]

Thus, our data in dwarf rats suggest that moderate (but not severe) suppression of GH contributes to lifespan extension. This finding contrasts with those in long-lived mice whose GH signaling is almost deficient [15, 16].

CR increased the lifespan in all rat groups. Although CR decreased the plasma IGF-1 level in each rat group compared with the corresponding AL group, the reduced IGF-1 level alone is unlikely to contribute to the extended lifespan. As described above, the lifespan in (tg/tg)-AL rats was shorter than that in (tg/–)-CR rats, even if leukemic death was eliminated, while the plasma IGF-1 level was similar between the groups. Therefore, CR could have a GH-IGF-1 independent mechanism(s) for lifespan extension.

3 Subpopulation of Thymic and Splenic Lymphocytes

3.1 Thymic Lymphocyte Subpopulation

The weight of the thymus at 6 months of age did not differ significantly between rats or between diet groups, when normalized for body weight (Table 3). Flow cytometric analysis of thymocytes prepared from the 6-month-old rats illustrated that only double negative (DN; CD4– & CD8–) cells tended to increase in (tg/–) and (tg/tg) rats, particularly in (tg/–)-CR rats. However, there was no significant difference in double positive (DP; CD4+ & CD8+), CD4-single positive (SP4), or CD8-single positive (SP8) subpopulations among rats or between diet groups. Thus, the present data suggest that the suppression of GH or CR does not significantly affect the composition of thymocytes at least in the 6-month-old male rats.

The present data in Tg rats are in accord with the dwarf mice models in which pituitary GH, PRL, TSH are deficient or IGF-1-null mice, or hypophysectomized mice demonstrating no statistical difference in thymocyte subsets between the hormone-deficient mice and their normal littermates [6]. Neither GH nor IGF-1 is required for primary lymphopoiesis. However, possible aging-related changes in thymic cell subpopulations and functions in Tg rats need to be analyzed in future studies.

3.2 Splenic Lymphocyte Subpopulation and Mitogenic Response

The weights of the spleen did not differ between the rat groups when normalized for body weight (Table 4); the normalized weight was reduced by 4–9% by CR. The proportion of T-cells (CD3+/CD45R–) did not differ among rat groups, while it was slightly increased by CR, particularly in (–/–) rats. The B cell population (CD3–/CD45R+) was lower in (tg/tg) rats; CR significantly decreased the B cell population. Subsequently, the T/B cell ratio did not differ among rat groups, although it was increased in the CR group, particularly in (–/–) and (tg/tg) rats. The NK cell

Table 3 Subpopulation of thymocytes in the transgenic and calorie-restricted rats at 6 months of age

	(–/–)		(tg/–)		(tg/tg)		
	AL	CR	AL	CR	AL	CR	2-f ANOVA
Thymus	40.4 (4.4)	47.0 (4.8)	51.5 (6.6)	39.0 (6.6)	43.0 (3.2)	33.3 (1.4)	
DN	2.8 (0.4)	3.0 (0.6)	3.2 (0.7)	6.1 (0.6)*/#	4.5 (1.1)	4.4 (0.6)	Genotype effect, p = 0.0508
DP	73.6 (1.5)	75.7 (2.4)	76.4 (2.0)	72.9 (2.4)	76.5 (3.8)	75.2 (1.0)	
SP4	20.3 (3.0)	18.1 (1.4)	17.7 (1.5)	18.0 (2.0)	16.2 (2.4)	17.4 (1.3)	
SP8	3.3 (0.3)	3.2 (0.5)	2.7 (0.3)	3.0 (0.3)	2.8 (0.5)	3.1 (2.3)	

Values represent the means (standard error) of 5 or 6 rats. Thymus (mg/100g body weight); DN, DP, SP4, SP8 (% of total thymocytes). (–/–); wild type rats. (tg/–); transgenic hemizygotic rats. (tg/tg); transgenic homozygotic rats. AL; ad libitum feeding rats. CR; 30% calorie-restricted rats. * $p < 0.05$ versus (vs) the AL group in each genotype. # $p < 0.05$ vs (–/–) in each diet group.

(CD3–/NKR+) population and activity were decreased in (tg/tg)-AL rats compared with (–/–)-AL rats. The proportion of NK cell but not the activity of NK cells was increased in the CR group.

The naive T-cell (CD4+, OX22+) population was greatest in the following order; (tg/tg), (tg/–), and (–/–) rats. Memory T-cell (CD4+, OX22–) population was slightly reduced in (tg/tg) rats. CR also increased the proportion of naive T-cells, particularly in the (–/–) rats.

The mitogenic response of splenic cells, examined by the response to phytohemagglutinin (PHA), concanavalin (CON) A, and anti-CD3 antibodies did not differ significantly among groups (Table 5); responses to PHA and ConA tended to increase in CR groups.

Thus, it can be summarized as follows: 1) that reduction of GH does not affect the T and B lymphocyte populations except NK cells, 2) severe suppression of GH decreases the cell number and activity of NK cells, 3) CR increases the T-cell fraction and decreases B cells in splenic cells, 4) CR restores the NK cell function that was reduced by severe suppression of GH, 5) reduction of GH does not affect the proliferative response of splenic lymphocytes to stimulants, while CR tends to enhance this response.

Although our analysis is limited to 6-month-old rats, our results suggest that CR and suppression of GH affect the development of secondary lymphoid organs. The aged immune system is characterized by a decrease in T-cell function caused by an increase in the fraction of memory T-cells that are less capable of responding to mitogens or novel antigen stimulation [17, 18]; in contrast, the number or proportion of naive T-cells declines. Thus, we can speculate that the increased proportion of naive T-cells in the CR group and (tg/–)-AL rats is attributable in part to the prolonged lifespan. The restored NK cell activity and/or increased T-cell fraction by CR in (tg/tg) rats might also contribute to the extended lifespan in short-lived (tg/tg) rats, because these innate immune functions have important roles for the prevention of cancers [19].

Our analysis suggests that CR and moderate GH suppression exert similar beneficial effects on immune function, while severe suppression of GH may produce some adverse effects.

Table 4 Subpopulations of splenic lymphocytes

	(–/–)		(tg/–)		(tg/tg)		
	AL	CR	AL	CR	AL	CR	2-f ANOVA
Spleen (g)/ 100 g Body weight	0.19 (0.04)	0.19 (0.02)	0.20 (0.01)	0.18 (0.01)	0.19 (0.01)	0.17 (0.01)	CR effect, p = 0.0477
CD3+ (%)	40.5 (4.2)	49.2 (1.7)*	44.3 (2.2)	42.2 (1.8)#	40.8 (1.7)	45.7 (1.6)	CR effect, p = 0.0449
CD45R+ (%)	45.1 (4.0)	34.7 (2.9)*	42.7 (2.1)	39.1 (3.1)	39.2 (1.4)	29.7 (1.2)*	GH effect, p = 0.0341 CR effect, p = 0.0007
T/B cell ratio	0.97 (0.22)	1.50 (0.18)*	1.06 (0.10)	1.13 (0.12)	1.06 (0.08)	1.55 (0.08)*	CR effect, p = 0.0024
Naïve T (%)	13.5 (1.1)	18.2 (1.0)*	18.0 (0.6)#	21.0 (1.1)	21.1 (1.4)#	23.9 (2.4)#	GH effect, p = 0.0003 CR effect, p = 0.0049
Memory T (%)	15.9 (0.4)	15.4 (0.7)	15.9 (1.0)	13.8 (0.4)*	12.9 (0.9)#	12.2 (0.5)#	GH effect, p = 0.0003 CR effect, p = 0.0573
N/M ratio	0.85 (0.08)	1.20 (0.09)	1.15 (0.08)	1.52 (0.06)*	1.68 (0.15)#	1.99 (0.22)#	GH effect, p < 0.0001 CR effect, p = 0.0031
NKR+/CD3- (%)	7.5 (0.7)	7.8 (0.6)	7.0 (0.5)	9.7 (0.8)*	5.8 (0.2)	9.9 (1.0)*/#	CR effect, p = 0.0002 GH x CR interaction, p = 0.0285
NK cell activity (%)	37.7 (3.6)	36.2 (3.0)	34.8 (3.3)	35.9 (3.9)	27.1 (1.0)#	35.3 (3.9)	

Values represent the means (standard error) of 5 or 6 rats. (–/–); wild type rats. (tg/–); transgenic hemizygotic rats. (tg/tg); transgenic homozygotic rats. AL; ad libitum feeding rats. CR; 30% calorie-restricted rats. * $p < 0.05$ versus (vs) group AL in each rat group. #, $p < 0.05$ vs (–/–) in corresponding diet group.

Table 5 Proliferative response of splenic cells

	(–/–)		(tg/–)		(tg/tg)		
	AL	CR	AL	CR	AL	CR	2-f ANOVA
PHA	2.69 (0.81)	4.87 (1.62)	4.57 (0.99)	6.11 (1.41)	2.96 (0.71)	6.43 (1.97)	CR effect, p = 0.0285
ConA	7.12 (0.4)	8.88 (1.49)	7.01 (0.78)	9.58 (1.15)	6.97 (0.69)	11.02. (2.68)	CR effect, p = 0.0276
aCD3	4.63 (0.62)	4.41 (0.72)	5.34 (0.95)	6.21 (0.95)	4.16 (0.66)	6.27 (1.51)	
LPS	5.09 (0.45)	5.12 (0.42)	3.88 (0.53)	6.01 (0.83)*	4.40 (0.34)	5.13 (1.26)	

Values represent the means (standard error) of 5 or 6 rats. Proliferative response (stimulation index) of splenic cells to PHA, ConA, antibody to CD3 (aCD3), and lipopolysaccharide (LPS) was evaluated as previously reported {Utsuyama, 1997 #103}. (–/–); wild type rats. (tg/–); transgenic hemizygotic rats. (tg/tg); transgenic homozygotic rats. AL; ad libitum feeding rats. CR; 30% calorie-restricted rats. * $p < 0.05$ versus (vs) the AL group in each genotype.

4 Response to LPS-induced Inflammatory Challenge

CR protects laboratory rodents against a variety of stressors including inflammatory and toxic agents [20]. Many stressors damage cellular components through an increase in reactive oxygen and nitrogen species, i.e., oxidative stress, which are thought to cause or accelerate aging and diseases. Thus, resistance to stressors could be one of the essential mechanisms underlying the retardation of aging and prolonging the lifespan of organisms. Indeed, embryonic or skin fibroblasts prepared from long-lived mouse models have been shown to resist oxidative stress induced by UV light, hydrogen peroxide, paraquat, or heavy metals [21]. Some of the mice models also exhibit higher survival rates after paraquat administration [22, 23].

We analyzed the acute phase response of 6-month-old Tg and CR rats to lipopolysaccharide (LPS), a component of Gram-negative bacteria that elicits inflammatory processes. LPS initiates a cascade of cytokine mediators, i.e., successive waves of increments of the plasma concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 [24]. The initial step of activation of cytokines subsequently augments secretion and synthesis of interferon (IFN)-γ, an incremental increase in nitric oxide (NO) by induction of iNOS and platelet-activating factor in the plasma, and increased synthesis of acute phase reactants. In the activation cascade, monocytes and macrophages are functionally enhanced to eliminate invading bacteria; however, these processes also result in endothelial cell injuries, which, in turn triggers the coagulation process, and finally lead to hypoperfusion and ischemic injuries in peripheral tissues.

The procedure of LPS-induced inflammatory challenge was described in more detail elsewhere [25]. Briefly, a low dose of LPS (1.6 mg/kg body weight) administered intraperitoneally significantly increased the blood AST (aspartate aminotransferase) level, an indicator of tissue injury, at 4 and 8 h in control

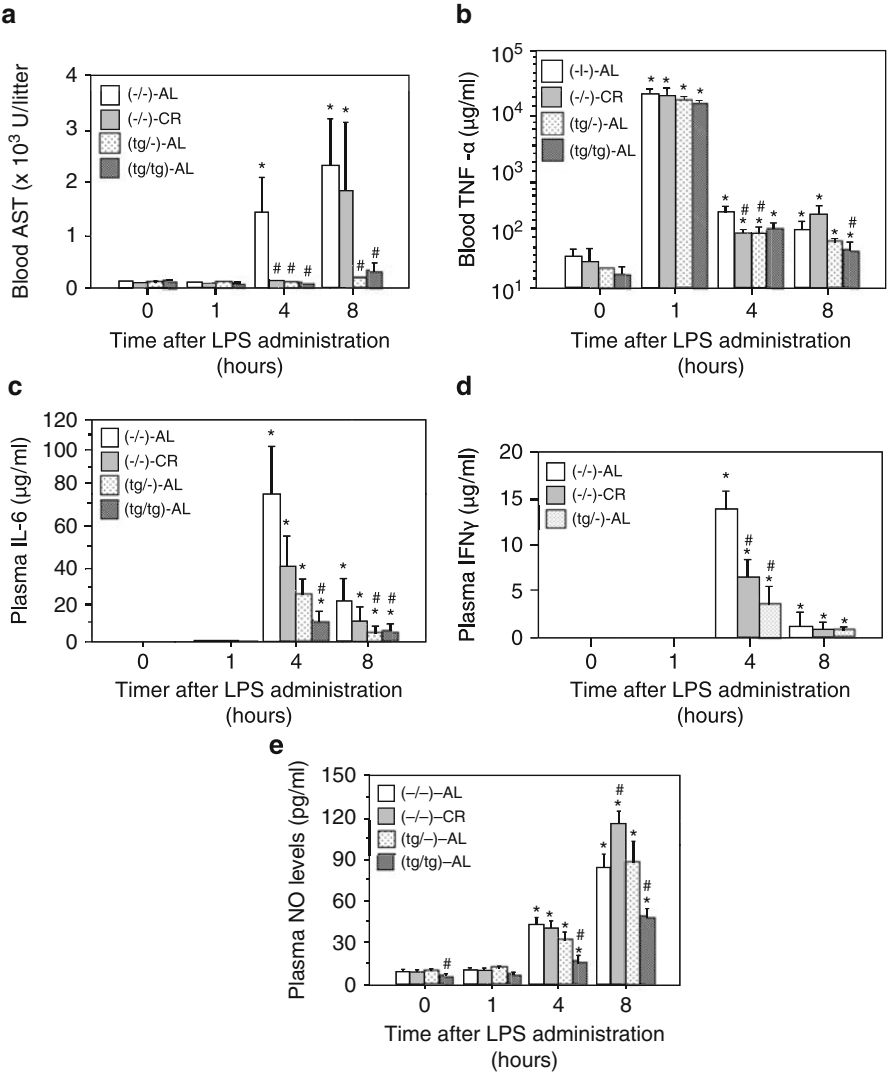

Fig. 3 **(a-e**) Response to LPS-induced inflammatory stress in CR and transgenic dwarf rats at 6 months of age. Values represent means + SE of 3–8 rats. * $p < 0.05$ versus 0 h in each rat group; # $p < 0.05$ versus (–/–)-AL rats at each time point. Blood or plasma samples were prepared for the following enzyme, cytokines, and nitric oxide assays (refer to Tsuchiya T et al [23] for further details) a) Blood levels of aspartate aminotransferase (AST), an index of tissue injuries after LPS administration. b) Tumor necrosis factor (TNF)-α, c) Interleukin (IL)-6, d) Interferon (IFN)-γ e) Nitric oxide (NO)

(–/–)-AL rats (Fig 3a). CR delayed the incremental increase in blood AST. In (tg/–)-AL and (tg/tg) rats, there was no significant increase. These findings indicate that either moderate or severe suppression of GH diminishes tissue injuries due to LPS

challenge. This effect seems to be stronger than that of CR. Since the degree of suppression of the GH-IGF-1 axis, indicated by the plasma concentration of IGF-1, was greater in (tg/–)-AL rats than in (–/–)-CR rats, the levels of AST correlated with the GH-IGF-1 levels.

The blood TNF-α did not differ between (–/–)-AL and (–/–)-CR rats at 1 h (Fig 3b); however, the TNF-α level at 1 h was low in (tg/–)-AL and (tg/tg)-AL rats. IL-6 was significantly increased at 4 h and reduced at 8 h (Fig 3c). This level was lower in the following order; (tg/tg)-AL, (tg/–)-AL, (–/–)-CR, and (–/–)-AL rats. The peak values of plasma INF-γ at 4 h was lower in the following order; (tg/–)-AL, (–/–)-CR, and (–/–)-AL rats (data for (tg/tg)-AL rats are not available), the finding was comparable to those of AST and IL-6. The level of NO was gradually increased between 0 and 8 h (Fig 3d). The level at 8 h was highest in (–/–)-CR rats and similar between (–/–)-AL and (tg/–)-AL rats, although the level was lowest in (tg/tg)-AL rats. These findings suggest that the suppression of GH attenuates the LPS-induced cytokine activating cascade and minimizes tissue injuries, and that CR also diminishes tissue injuries probably, in part, through the same mechanism, because CR modestly suppressed the GH-IGF-1 axis. The difference in severity of tissue injuries between the rat groups seemed to correlate with the degree of suppression of GH-IGF-1 axis. CR, however, could affect the LPS-initiated inflammatory cascade and related tissue injuries differently, because the NO level at 8 h was significantly higher in (–/–)-CR rats than in (–/–)-AL and (tg/–)-AL rats.

Previous studies indicate that GH primes phagocytes for an increased production of reactive oxygen intermediates [26, 27]. GH potentiates the biological activities of entotoxin, i.e., lethality, in the rat [28]. However, IGF-1 did not induce this effect, indicating that the enhancement of endotoxin effects by GH is via an IGF-1-independent pathway [29]. Priming rats by GH induced a further increased response to serum IFN-γ but not TNF-α to subsequent entotoxin challenge, suggesting that INF-γ rather than TNF-α is likely to be involved in this process [29]. In other words, the suppression of GH diminishes the propagation of the inflammatory cascade downstream of TNF-α and minimizes tissue injuries. In this context, we can conclude that lower levels of GH favors longevity in animals via minimization of activation of monocytes and macrophages that are provoked by inflammation at the molecular levels during the aging process.

The diminution of inflammatory cascade could sometimes be harmful in organisms particularly regarding the elimination of invading bacteria. GH-treated animals release more superoxide and TNF-α in response to the appropriate trigger stimuli and ingest Listeria monocytogenes better than macrophages from untreated animals [30, 31]. GH has also been shown to protect hypopituitary animals from lethal *Salmonella typhimurium* infections [31]. In nature, infectious challenges by microorganisms are frequent and, thus, enhanced innate immunity could be beneficial to increase survival, even if the enhanced immunity also damages host tissues and cells. Therefore, there could be optimal levels of the strength of innate immunity in animals to protect infectious agents but minimize host–tissue damage, depending on their living conditions.

5 Conclusion

If the GH levels in commercially available rats are set as control values, moderate suppression of GH favors longevity and exhibits few demerits in the immune system. Severe suppression of GH may have some adverse effects on innate immunity, e.g., the diminished NK cell activity and the attenuated cytokine activation cascade that may decrease survival probability under usual living conditions where animals are often exposed to a variety of infection agents. In other words, there could be optimal levels for GH to maximize survival of organisms, depending on living conditions. Under SPF conditions in the laboratory, the demerits of severe suppression of GH are masked and only the merit, minimization of host-tissue injuries caused by the self-defense system, is emphasized.

Tg and CR rats exhibited similar trends regarding the selected immune functions, suggesting that the GH signaling could mediate the effect of CR in part. In this sense, the Tg rat is an intriguing animal model to better understand the roles of the GH axis in the aging process and the anti-aging effect of CR. Because our analysis on the immune system in Tg rats is limited, future studies are required to further understand the role of immunosenescene in the aging process and the anti-aging effect of CR.

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Mathematical Modeling of Immunosenescence: Scenarios, Processes and Limitations

A.A. Romanyukha, S.G. Rudnev, T.A. Sannikova and A.I. Yashin

Contents

Abstract: Mathematical modeling of immunosenescence is the new area of research emerging at the interface of the immunology, gerontology, and mathematics. In this paper we outline basic variables important for modeling aging immunity. We discuss the role of evolution in shaping pattern of aging in the immune system of modern humans. We investigate mathematical models of postnatal changes in the population of peripheral T-cells, effects of the antigenic load during development on the body growth, and contribution of immunosenescence to the old age increase in the risk of death from respiratory infections.

Keywords: Antigenic load • aging immunity • mortality from infections • body growth • population of T-cells

1 Introduction

There are two types of mathematical models applied to the life science problems. The objectives and methodology used in these models differ substantially. The first type of models deals with the problems of analysis and interpretation of the results

A.A. Romanyukha (✉) · S.G. Rudnev · T.A. Sannikova
Institute of Numerical Mathematics
Russian Academy of Sciences, Moscow, Russia

A.I. Yashin
Center for Population Health and Aging
Duke University, Durham, USA

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of a certain experimental study, or a series of such studies. The main objective of such modeling is the quantification of sensitivity of the phenomenon to changes in various factors. The results of modeling contribute to better understanding the roles of factors and mechanisms in the processes under study. The focus of the second type of models is systematization of knowledge and data in order to develop systemic view, obtain integral description of the results of heterogeneous experimental studies, and check consistency of such description with existing theories. The models of the second type deal with the description of the phenomenon: they assimilate results of different experiments, test their mutual compatibility, and correspondence to existing theories. Such models are an effective means of testing accumulated knowledge and can be used to predict effects of exposure to external factors on functioning of living systems, or changes in characteristics of the organism itself.

2 Modeling Immunosenescence

In this study we will investigate properties of the first type models of aging immunity. Such modeling is a relatively new area of research, emerging at the interface of immunology, gerontology, and mathematics. It studies regularities of aging related decline in functioning of the components of the immune system, as well as dynamic interaction among them during the aging process. To describe such nonlinear multidimensional aging related changes in the immune defense mechanism the dynamic mathematical and computer modeling of these phenomena is needed. An important step in such modeling is the selection of variables or "units" of the immunosenescence. These units suppose to reflect the basic features and processes of aging immunity. These variables include:

- The characteristics of the lymphocytes' aging (telomere length, the ability to respond to the antigenic and cytokine signals, intercellular cooperation;
- Population-wide characteristics of lymphocytes and other immune system cells (proportion of naïve and memory cells, the proportion of the various lymphocyte subpopulations, antigenic repertoire of lymphocytes);
- The characteristics of activity of the immune system (rate of lymphocyte formation in thymus and the bone marrow, the amount of active parenchyma in the lymphoid organs, the rate of lymphoid proliferation processes);
- The characteristics of the state of the organism (frequency and severity of infectious disease, activity of inflammatory process, probability of death and/or decrease of reproduction due to the deficiency of immune protection).

2.1 The Immune Life Histories

Thus, the units of immunosenescence should relate lymphocytes' characteristics to the processes developing in the aging body. These include infection, inflammation, tissue and organ aging, fertility and life span. These variables should relate the dynamics of lymphocyte populations and characteristics of individual fitness. The

age trajectories of these interrelated variables comprise the immune life history (McDade 2003). We will consider age-related changes in the: naïve cells concentration; memory cells concentration; replicative potential of lymphocyte subpopulations; antigen repertoire of lymphocytes; rate of the influx of immune cells from the thymus and bone marrow; incidence of infectious and, maybe, cardiovascular diseases; individual inflammatory status; antigenic load. All values, except the latter are characteristics of the immune system. The antigenic load is a measure of pressure of external and internal conditions at the immune system.

2.1.1 Antigenic Load

We define the antigenic load as the rate of the inflow of the alien, or modified antigens into the lymphoid tissue. The activity of the immune system and its rate of aging depend significantly on the level of antigenic load, because it affects the rate of division of the lymphocytes and their mortality risk. The antigenic load consists of the two components: alien antigens and modified self-antigens. Alien antigens may arrive from the external environment and reproducing in the host. They may also be located in the host and reproducing in certain circumstances.

The rate of inflow of infectious and noninfectious antigens from the environment must be proportional to the consumption of nutrients and the oxygen. Because such consumption is directly connected to the metabolic rate, it is reasonable to hypothesize that the level of external antigenic load is proportional to the metabolic rate.

A large proportion of modified self-antigens are formed due to action of free radicals; the rate of their formation is proportional to the metabolic rate as well. So we will assume that the rate of generation of self-antigens is proportional to the metabolic rate. Therefore, the total antigenic load largely depends on the intensity of metabolism.

The level of infectious component of the antigenic load is strongly influenced by the two factors, weakly dependent on the metabolic rate: the density of infectious microorganisms in the environment and the effectiveness of the immunity. Obviously, the high density of microorganisms in the environment increases the frequency of infections and, hence, the antigenic load also increases. On the other hand, in the presence of many memory cells, the infectious antigenic load will loosely depend on the microbial density or metabolic rate.

2.1.2 Antigenic Load and Immunosenescence

The intensity of metabolism declines with age. The effectiveness of the immune protection also varies with age: the frequency of infectious diseases is highest in the childhood, lowest in the intermediate age and increases in the old ages. Specific characteristics of the antigenic load affect the development of the immune system, its aging rate, and, hence, individual fitness. Consequently, many properties of the aging immunity, observed in modern humans, are formed by evolution during many thousands of years in the past. The principal objectives of the immune system are to ensure survival to reproductive age and provide maximum protection against infections during the reproductive period.

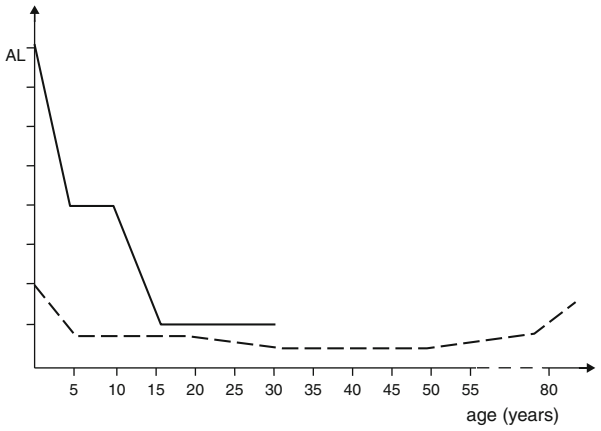

Fig. 1 Hypothetical scheme of life-long antigenic load dynamics for primitive (—), and contemporary man (— —), AL—antigenic load (in arbitrary units)

An important mechanism for ensuring effective protection involves adaptive immunity based on production of the immune memory cells. The more memory cell is formed after the primary immune response, and the longer they live; the more protected is the body from secondary infections of this type. Since the resources of the immune system are limited, there is a competition between the naïve cells and memory cells. The memory cells provide effective protection from a few known pathogens in current and future situations. The naïve cells are supposed to provide future protection from all possible pathogens. If the life expectancy is large and emergence of the new diseases is a likely scenario, it is beneficial to maintain more naïve cells and have active thymus, with less effective protection against endemic pathogens. In case of short life and low rate of emergence of the new diseases it is more profitable for an organism to produce less naïve lymphocytes and make higher investment to the memory cells. Thus, the fundamental property of the immunosenescence is its evolutionary coadaptation with antigenic load and other life history characteristics of the aging human organisms.

Figure 1 shows a hypothetical dynamics of antigenic load during life of primitive and modern man. It is important to understand how the immune system, evolutionary adjusted to the living conditions of primitive man, adapts to current environmental and living conditions. The main difference between the curves 1 and 2 involves significant reduction of the infectious burden, especially at the beginning of life and an increase of life expectancy in modern humans[1].

2.2 Scenarios of Immunosenescence

The differences in the current and prehistoric antigenic loads affect process of immunosenescence. The age related changes in the immune system of a primitive human were formed by high infectious load in the first years of life, where the immunity learning period is included in the child's growth interval. Short life and the relative isolation

[1] When creating the Fig. 1 we have assumed that the antigenic load of the modern humans is approximately equal to load by their self-modified antigens, and the antigenic load of the primitive humans was about an order of magnitude larger than of the modern ones.

from the new pathogens defined the replacement rate of the naïve cells by the memory cells, the rate of reduction of the naive lymphocytes production, the rate of reduction of replicative capacity of the memory cells with age. An exposure to the antigens during the growth period ensured training of the immunity for survivors, efficient use of the body resources and protection against antigens during the reproductive period.

Note that the high infant mortality rate observed in the past may indicate substantial variability in the antigenic load among survivors. Such scenario of developing immunity can be called **forced immunomaturation**. The essence of this scenario is the accelerated maturation and learning of the immune system. Reducing the antigenic load at this scenario improves the immunity condition in middle age. In modern conditions external antigenic load has much less impact on the aging immunity. The age decline of immunity is determined by the changes in the stem cell properties, lack of naive cells, a narrow repertoire of the memory cells, and reduced replicative ability of the memory cells. Thus, in modern conditions immunosenescence is largely defined by traits selected during the evolution of immunity (early decline of naive cell production, reduction of their replicative ability, etc.) as well as by traits depending on general properties of aging body (the aging of stem cell pool, increase of the self-modified antigen generation rate, etc.). Such scenario of age related changes in immunity can be called **inertial immunosenescence.** An important feature of this aging scenario is that the decline in the antigenic load, or slowing down of thymus involution has little impact on immunity in the older ages.

In this case in order to improve the immune function a combined influence on the immune system such as the rejuvenation of stem cells, thymus function enhancement, accelerated elimination of the old memory and naive cells, and accelerated immunity learning through vaccination is required. This procedure will be accompanied by a temporary decrease in the immune protection. Therefore, this process can not be conducted on the background of a strong decline in immunity, the optimal age is the border between medium and older age.In fact this is a repetition of immunity development and learning period in its reduced form. It can be assumed that in some individuals such events can occur as a result of natural events (starvation, severe stress, etc.).Hypothetical version of immune life history with such periodic recovering of the immune system can be called the **reciprocating immunorejuvenation** scenario.

2.3 Constraints of Adaptation

The adaptation responses develop in the presence of explicit or implicit limitations on the rate and the magnitude of physiological processes involved in such adaptations. For example, the total number of immune system cells should not change significantly during the adulthood. This restriction results in diminishing the memory cell life time, when the production rate of the naive cells by the thymus increases. An example of implicit limitation of the immune system adaptation is the need to coordinate the growth of the body size, growth of the immune system mass, and the rate of the immunity learning. If the new antigens presentation will delay an increase in the body mass the homeostatic proliferation of lymphocytes may lead to undesirable distortions in the immune cells' repertoire.

Mathematical modeling is a convenient method for studying such problems. Below we will consider examples of the use of mathematical models to study aging related changes in the immune system. They include postnatal changes in the population of peripheral T-cells, effects of the antigenic load during development on the body growth, and contribution of immunosenescence to the old age increase in the risk of death from respiratory infections.

First we investigate how the basic immunosenescence processes such as thymus decay, shortening of telomeres in the newly forming naive T-lymphocytes and shrinking of the peripheral lymphoid tissues interact depending on the antigenic load.

For description of these processes, we propose a mathematical model (1). It describes the balance of influx and usage of T-cells and their replicative potential. The model equations are based on two main assumptions:

- the T-lymphocyte concentration in the peripheral lymphoid tissue must be maintained constant;
- the naive cells are superior to the memory cells in the competition for free space in the lymphoid tissue.

Based on these assumptions, we construct a model describing the age dynamics of the following variables:

N(t),* rate of naive T-cells influx in IPLT at the age t (cell/day);
V(t), volume of IPLT at the age t, (ml);
P(t)*, length of telomere repeats in naive T-cells produced at the age t, (bp/cell);
N(t), concentration of nai'ye T-cells in IPLT at the age t, (cell/ml);
M(t), concentration of memory T-cells in IPLT at the age of t (cell/ml);
PN(t), average length of telomere repeats in naive T-cell at the age t (bp/cell);
PM(t), average length of telomere repeats in memory T-cell at thew age t (bp/cell);
Function *L(t)* describes total antigenic load at the age t (g/day).

The mathematical model of age-related changes in peripheral T-cell population is represented by the system of the following seven ordinary differential equations:

$$
\begin{aligned}
&\frac{dN^*}{dt} = -k_T N^*,\\
&\frac{dN}{dt} = \frac{N^*}{V} - \alpha_1 \frac{L}{V} N - \mu_N N - \frac{dV}{dt}\frac{N}{V},\\
&\frac{dP_N}{dt} = \left(P^* - P_N\right)\frac{N^*}{NV},\\
&\frac{dM}{dt} = \rho_1\alpha_1 \frac{L}{V} N + \rho_2\alpha_2 \frac{L}{V} M + \mu_M \left(C^* - N - M\right) - \frac{dV}{dt}\frac{M}{V},\\
&\frac{dP_M}{dt} = \rho_1\alpha_1 \frac{L}{V}\left(P_N - \lambda_N - P_M\right)\frac{N}{M} - \left(\rho_2 + 1\right)\alpha_2\lambda_M \frac{L}{V}.\\
&\frac{dV}{dt} = -k_V V,\\
&\frac{dP^*}{dt} = -k_p P^*
\end{aligned}
\qquad (1)
$$

For simplicity, we assume that the antigenic load remains constant throughout life, but the production rate of naive T-lymphocytes and the volume of peripheral lymphoid tissues decrease with constant relative rate. However, developed model is flexible enough to investigate more complicated scenarios.

Numerical experiments with this model revealed some interesting dependencies: the lengthy production of naive T-lymphocytes strengthens the immune protection in advanced ages, but relatively weakens immunity in middle age because it reduces maintenance resources and duration of immune memory. The calculations also showed that an important factor in ensuring the immune protection in advanced ages is slowing of the stem cell aging.

These results allowed for addressing the question on how the body growth processes affect the aging of immunity.

3 Modeling Postnatal Changes in the Population of Peripheral T-cells

The most apparent changes in the population of peripheral T-cells in humans occur in childhood (Rufer et al. 1999; Zeichner et al. 1999), when the relative rate of body growth and the infection morbidity are maximal. These changes are accompanied by the early onset of thymus atrophy—a primary lymphoid organ, in which the development of bone marrow-derived progenitors into mature T-cells takes place (Steinmann et al. 1985) (Fig. 2). Such atrophy substantially restricts the ability of adults to produce naive T-cells, which, in turn, affects the strength and efficiency of adaptive immune response. An expansion of intact peripheral lymphoid tissue (IPLT) at early age by memory cells affects the immune system learning capacity at later ages. Therefore, when studying the immune system aging, it is important to take the conditions and regularities of the development of this system early in life into account.

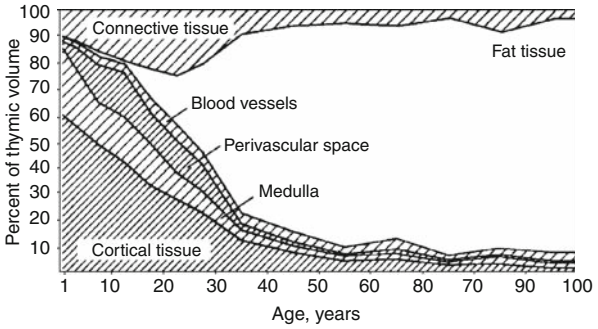

Fig. 2 Involution of thymus (Steinmann et al. 1985). After the age of 1, the volume of thymus remains relatively constant. The division of thymic precursor T-cells takes place primarily in cortical tissue

3.1 The Extended Model

We address these issues using the extended mathematical model of age-related changes in population of peripheral T-cells suggested by Romanyukha and Yashin (2003). The extended model adds one equation on age-related changes in body mass to the system of equations specified in (Romanyukha and Yashin 2003), and exploits the new fundamental assumption that the value of antigenic load is proportional to the intensity of basal metabolism. The resulting model allows for describing development of adaptive immunity during all postnatal life, including childhood. The dependence of basal metabolism on body mass is described using the Kleiber's 3/4 power scaling law (Kleiber 1932; West, Brown 2005).

Taking into account the above considerations, the mathematical model of age-related changes in population of peripheral T-cells can be written in the form:

$$
\begin{aligned}
&\frac{dN^*}{dt} = -k_T N^*,\\
&\frac{dN}{dt} = \frac{N^*}{V} - \alpha_1 \frac{L}{V} N - \mu_N N - \frac{dV}{dt}\frac{N}{V},\\
&\frac{dM}{dt} = \rho_1\alpha_1 \frac{L}{V} N + \rho_2\alpha_2 \frac{L}{V} M + \mu_M (C^* - N - M) - \frac{dV}{dt}\frac{M}{V},\\
&\frac{dP^*}{dt} = -\left(\frac{\bar{k}_P}{m}\frac{dm}{dt} + k_P\right) P^*,\\
&\frac{dP_N}{dt} = (P^* - P_N)\frac{N^*}{NV},\\
&\frac{dP_M}{dt} = \rho_1\alpha_1 (P_N - P_M - \lambda_N)\frac{L}{V}\frac{N}{M} - (\rho_2 + 1)\alpha_2\lambda_M \frac{L}{V},\\
&\frac{dV}{dt} = \alpha_3 \frac{L}{V}\frac{dm}{dt} - k_V V,\\
&\frac{dm}{dt} = \alpha_4 m^{3/4} - k_m m.
\end{aligned}
\tag{2}
$$

Here the variable t corresponds to individual's age; $N^*(t)$ is the rate of naive T-cells influx from thymus into IPLT; $N(t)$ is the concentration of naive T-cells in the IPLT; $M(t)$ is the concentration of the memory T-cells in the IPLT; $P^*(t)$ is the length of telomeres in naive T-cells leaving thymus at the age t; $P_N(t)$ is the length of telomeres in the naive T-cells; $P_M(t)$ is the length of telomeres in the memory T cells, $V(t)$ is the volume of the IPLT; $m(t)$ is the body mass. Rapid telomere shortening in the stem cells during the first years of life entails similar changes in telomeres' length of the newly produced naive T-cells in the thymus (Rufer et al. 1999). We assume that the corresponding rate is proportional to the relative increase in the body mass. So, the rate parameter in the equation for P^* can be written as a function of age: $k_P(t) = \bar{k}_P(dm/dt)/m + k_P$ where k_P is taken from the original model of Romanyukha and Yashin (2003). We assume also that the rate of the early IPLT expansion is proportional to specific anti-

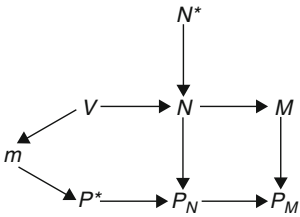

Fig. 3 The sequence of the model parameters adjustment

genic load (*L*/*V*) and the rate of body mass change. Initial conditions correspond to the age of birth:

$$N^*(0) = N_0^*, \quad N(0) = C^*, \quad M(0) = M_0^*, \quad P^*(0) = P_0^*,$$
$$P_N(0) = P_N^0, \quad P_M(0) = P_M^0, \quad V(0) = V_0, \quad m(0) = m_0. \tag{3}$$

The sequence of model parameters adjustment is shown in Fig. 3 Using this scheme, we constructed initial estimates of model parameters (Table 1).

Table 1 Initial parameters' estimates and initial conditions for simulation of age related changes in population of peripheral T-cells

Parameter	Physical meaning	Dimension	Value
α_1	Rate constant of naive T-cells stimulation	ml/g	1.5×10^{4}
α_2	Rate constant of memory T-cells stimulation	ml/g	1.5×10^{4}
α_3	Rate constant of the intact peripheral lymphoid tissue (IPLT) growth	$ml^2\times day/g$	3×10^{7}
α_4	Rate constant of body mass growth	$g^{1/4}/day$	2.5×10^{-2}
α_5	Parameter which relates antigen load and basal metabolic rate	$g^{1/4}/day$	2.8×10^{-10}
μ_N	Rate constant of natural death rate for naive T-cells	1/day	1.3×10^{-4}
μ_M	Rate constant of competitive death (or homeostatic proliferation) for memory T-cells	1/day	0.07
α_1	Number of memory T-cells produced by one naïve cell	—	100
α_2	Number of memory T-cells produced by one memory cell	—	1.1
λ_N	Length of telomere repeats lost during transformation of naïve T-cells to memory cell	base pairs (bp)	1400
λ_M	Length of telomere repeats lost during self-replication of memory cells	bp	500
C^*	Low limit for normal concentration of memory T-cells in intact lymphoid tissue	cell/ml	2.5×10^{9}
k_T	Rate of diminishing of naïve T-cells production with age	1/day	1.1×10^{-4}
k_V	Relative rate of reduction of the IPLT volume with age	1/day	2.7×10^{-5}
k_p	Relative rate of the telomere repeats reduction in the progenitor of naïve cells	bp/day	1×10^{-5}
k_P	Relative rate of accelerated telomere shortening in the progenitor of naïve T-cells in early childhood	bp/day	0.07
k_m	Rate parameter in the equation for body mass	1/day	1.5×10^{-3}
N^*_0	Rate of naive T-cells release from thymus at birth	cell/day	8×10^{8}
N^0	Concentration of naïve T-cells in the IPLT at birth	cell/ml	2.5×10^{9}
M^0	Concentration of memory T-cells in the IPLT at birth	cell/ml	2.5×10^{7}
P^*_0	Average length of telomeres in naive T-cells leaving thymus at birth	bp	10370
P^0_N	Average length of telomeres in naive T-cells in the IPLT at birth	bp	10370
P^0_M	Average length of telomeres in memory T-cells at birth	bp	8970
V_0	Volume of intact lymphoid tissue at birth	ml	150
m_0	Body mass at birth	g	3500

3.2 Parameters' Estimation

The level of agreement between the model and data was characterized by the value of the least-squares function for the log-transformed data and model solutions.

$$F(\alpha) = \sum_{i,j} \left(\lg \left(\frac{x^i(t_j, \alpha)}{X^i_j} \right) \right)^2$$

Here, α is the vector of model parameters, $x^i(t_j,\alpha)$ is the value of the i th component of model solution at age t_j, and X^i_j is the corresponding data. The solution of the constrained minimization problem of the function $F(\alpha)$ obtained on a subset of model parameters is shown as dotted lines on Fig. 4. It was obtained using differential evolution (DE) algorithm (Storn, Price 1997). The refined parameters are shown in Table 2. One can see from Fig. 4 that the model satisfactorily describes the data on age-related changes in T-cell populations at the entire interval of aging except for the initial age interval.

During the first 6 months of life, the immune defense is provided mainly by maternal antibodies though it was shown recently that the mature T-cell immune response against viral and macroparasitic infections may occur even in the prenatal conditions (King et al. 2002; Marchant et al. 2003; Hazenberg et al. 2004). Along with an increase in the volume of thymus, the total number of peripheral T-cells and the volume of the IPLT grow rapidly. Between ages of 0.5 and 6 years the number of lymphocytes in the body remains relatively stable and then increases, approaching a maximum at the age of 20 years (Valentin 2002). At the initial age interval a rapid decline in the length of telomeres of the newly produced T-cells in the thymus takes place (Rufer et al. 1999). These data were not fit well by the model (Fig. 4). In order to investigate the dynamics of relative antigen load and also of naïve T-cells division, we considered a refined model using explicit log-linear functions for N^*, P^* and V at the corresponding initial age intervals.

Table 2 The results of sequential parameters' estimation for modeling age related changes in population of peripheral T-cells. Permissible boundaries for the model parameters are shown as XVmin and XVmax. The values of the residual function F are shown in the last row

Parameter	XVmin	XVmax	Initial estimate	Refined estimate		
				1	2	3
N^*_0	4×10^8	10^9	8×10^8	8.34×10^8		
k_T	8×10^{-5}	2×10^{-4}	1.1×10^{-4}	1.06×10^{-4}		
α_4	0.01	0.04	0.025		0.023	
k_P	5×10^{-5}	2×10^{-5}	10^{-5}		1.3×10^{-5}	
k_P	0.01	0.1	0.07		0.06	
α_3	10^7	5×10^7	3×10^7		2.8×10^7	
α_1	5×10^3	5×10^4	1.5×10^4			10^5
μ_N	10^{-4}	10^{-2}	1.3×10^{-4}			5×10^{-5}
ρ_1	10	1000	100			2000
ρ_2	1	100	1.1			324
μ_M	0.001	0.1	0.07			8.7
F			0.32	0.28	0.26	0.25

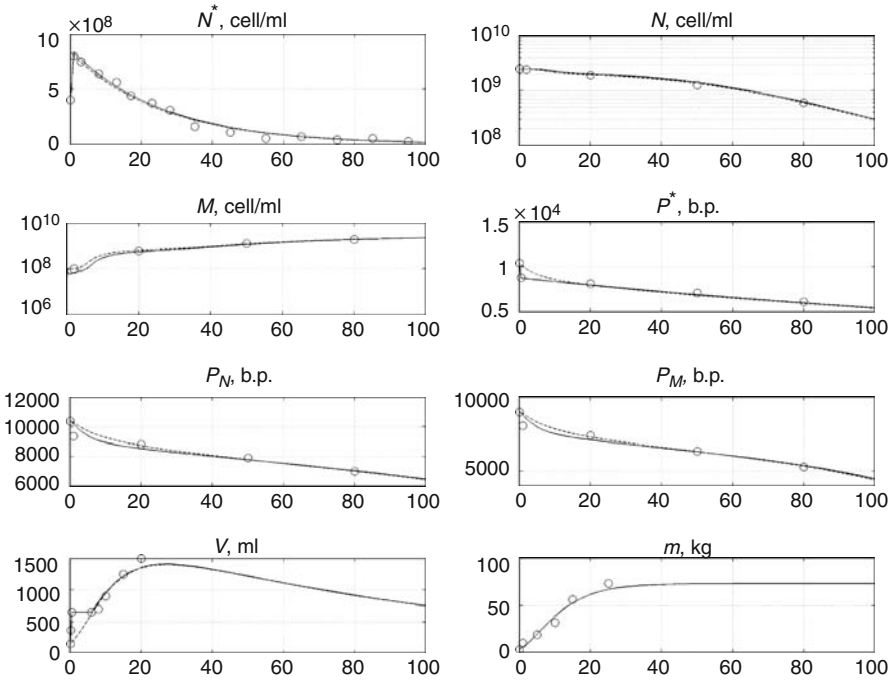

Fig. 4 Solution to the refined model system (solid lines). Dotted lines represent the solution to the initial model. Along the x-axis is age (years). The data are shown as open circles

The solution of the refined model is shown in Fig. 4 as solid lines. Because of the absence of parameters in the equation for P_N, it is interesting to see that the good agreement between P^* and the data turned out to be insufficient for the precise description of the telomeres' length in the naïve T-cells (P_N) early in life. One possible explanation involves the effect of the naive T-cells' *homeostatic proliferation* (Unutmaz et al. 1994). Such proliferation results in increased telomere shortening in the naive T-cells as compared with the rate induced by telomere shortage in the stem and/or precursor T-cells, and, hence, can be accounted for in the equation for P_N. One can see from the Fig. 4 that the rate of homeostatic proliferation of the naive T-cells is comparable with the rate of their production in thymus. Similar results were obtained in Hazenberg et al. (2000, 2004); Ye, Kirschner (2002); Dutilh, DeBoer (2003) when modeling data on the T-cell receptor excision circles (TREC) kinetics.

Figure 5 shows the dynamics of specific antigenic load L/V for the refined and initial models. The refined model is characterized by significantly smaller values of L/V at early ages. This can be interpreted as an initial "reserve" of the immune system owing to fast initial increase in the volume of the IPLT.

An important part of the antigenic load L is represented by the infection load, related to the impact of *multiplying* antigens. At present, it is difficult to obtain quantitative estimates of the relative contribution of the infection load to the total antigenic

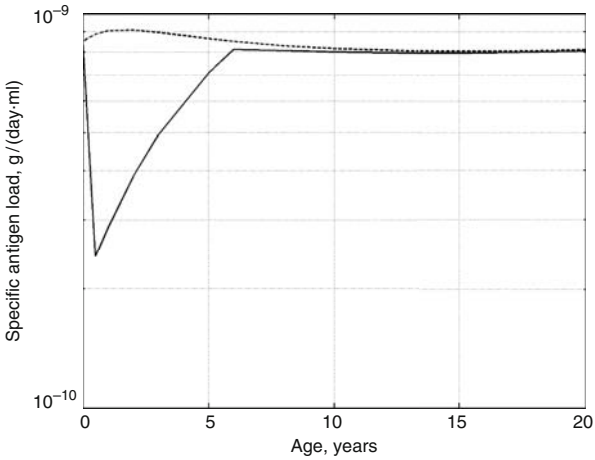

Fig. 5 Specific antigen load *L/V* as a function of age for refined (solid line) and initial models (dotted line)

load. However, the evidence is accumulating that this contribution can be significant. The results of simulation of the impact of the HIV infection on the rate of T-cell population aging suggest 2 to 8-fold increase in the fraction of divided naive T-cells, and, hence, of the total antigenic load (Hazenberg et al. 2000; Sannikova et al. 2004).

The magnitude of infectious pathogens growth in human body in case of acute infections vary from 10^5–10^6 for bacterial pneumonia (Romanyukha, Rudnev 2001) to 10^{10}–10^{11} for viral infections, such as influenza A, or hepatitis B (Marchuk et al. 1991; Bocharov, Romanyukha 1994).

We assume here that the total antigenic load L depends linearly from basal metabolic rate. The high rate of infection diseases in the childhood imply a significant excess of the value of L at this age compared to the value determined by the basal metabolic rate. One can assume that this "initial reserve" of the immune system is, in fact, "consumed" by the infection load. As a result, the specific antigenic load, L/V, can be significantly *higher* than the values suggested by the refined model (continuous line on Fig. 5). The comparison of graphs in Fig. 5 suggests that the initial reserve of the immune system early in life allows for 2–4-fold increase in the total antigenic load above the values permitted by the basal metabolic rate.

4 Immune System Development and Body Growth

The increasing body of evidence from animal and human studies supports the idea on the existence of trade-off between immune defense and organism's growth. For example, the data from gnotobiological studies show that the infection of germ-free chicken impairs body growth by 15–30% (Lochmiller, Deerenberg 2000). Primary immunodeficiencies in humans can also lead to growth impairment and even growth failure (Bjorkander et al. 1984). This holds true for the HIV infection, depending on

the extent of the viral load (Arpadi et al. 2000) which, presumably, reflects a rising energy deficit caused by the gradual increase in the antigenic load.

The suggested model allows for evaluation of possible consequences of such a trade-off. For this, we assumed a linear dependence of the body growth from the antigenic load: $\alpha_4 = a - b\alpha_5$, where α_4 is the rate constant of the body growth, and α_5 is the parameter, which relates antigenic load and basal metabolic rate. The parameter a in this formula characterizes a maximal rate of body growth attained in the absence of antigenic load, and b describes the detrimental effect of antigenic load on the body growth.

For illustrative purposes, based on the results of modeling the effects of HIV infection on the rate of immune system aging (Hazenberg et al. 2004; Sannikova et al. 2004), we assumed that the arrest of the body growth takes place when the value of antigenic load is 10-fold greater than normal. From this assumption, the values of a and b were determined. The results of calculations suggest the presence of stabilizing effect of the body growth on the immune system development: the antigenic load up to 1.5 times higher than normal insignificantly affects the dynamics of model variables except for the rate of body mass change and the stationary level of the body mass in the adulthood (changes from 73 kg to 60 kg). Further increase in the antigenic load results in a more pronounced effect on the immune system development with the remarkable effect on the volume of the IPLT at the adult age. Counter-intuitively, both an increase and decrease in the antigenic load result in the detrimental effect on the "adult" values of the volume of the IPLT with mild unidirectional effect on the dynamics of the naïve and memory cells and in the opposite effect on the telomeres' lengths. A decrease in the antigenic load results in the increased level of the adult body mass with a maximum of 115 kg in the absence of the antigenic load (Fig. 6).

The results presented in Fig. 7 show an increasing effect of antigenic load on body mass with age.

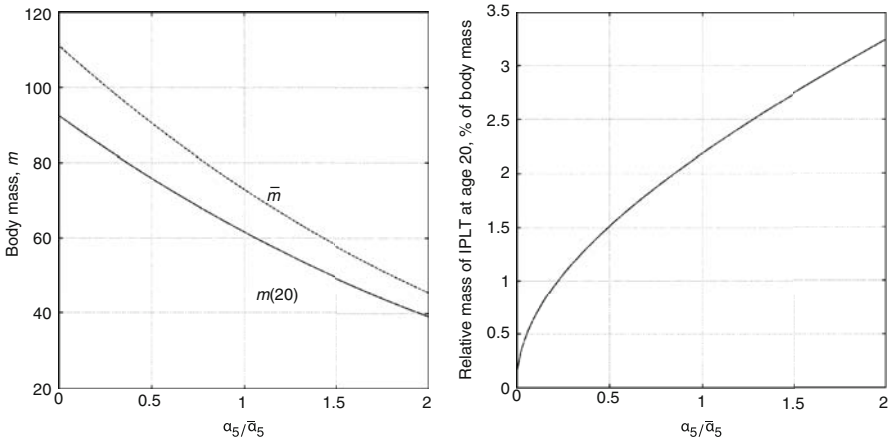

Fig. 6 The influence of antigenic load on body mass at age 20 (left panel), and on the relative mass of the IPLT (right panel)

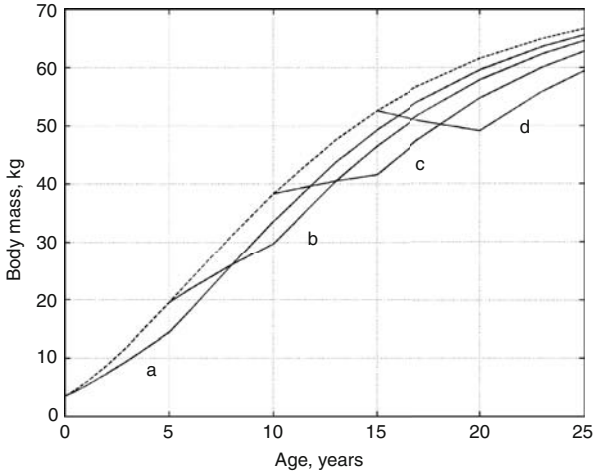

Fig. 7 The influence of 2-fold increase in antigen load at the age intervals (a) [0, 5 years], (b) [5, 10 years], (c) [10, 15 years] and (d) [15, 20 years] respectively, on the dynamics of body mass. The normal age-related changes of body mass are shown as dotted line

5 Model of Age-related Risk of Death from Respiratory Infections

Preliminary analysis revealed that mortality rates from pneumonia and other respiratory infections follow certain regularity pattern in different human populations (Sannikova 2007). The principal traits of the pattern are relatively high mortality level during infancy and early childhood, very low during the reproductive period, exponential (or faster) increase after age 50. Since such an increase takes place despite the presence of the modern health care systems we suppose that the aging of the T-cell immunity is responsible for the steep growth of pneumonia mortality curve at advanced ages.

We develop a mathematical model establishing the relationship between age-related changes in the peripheral T-cells population and mortality caused by respiratory infections (Fig. 8).

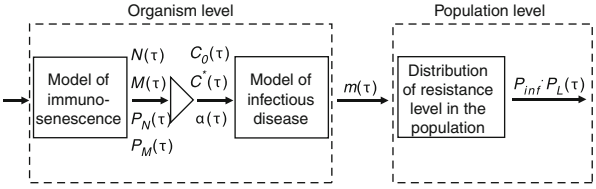

Fig. 8 The relationship between age-related changes in the peripheral T-cells population and increasing risk of death from infectious disease. The proliferative capacity of the T-cells decreases with age, which results in deceleration of lymphocyte proliferation during the immune response. So, the severity of the disease increases with increasing age. The higher the disease severity, the higher the risk of the lethal outcome

The model of age-related risk of death from respiratory infections consists of three component models: a model ofage-related changes in peripheral T-cells population (1), a model of infectious disease (Marchuk 1997) and a relationship between disease severity and risk of death. Numerical solution of the system (1) yields the sets of immune characteristics (such as the concentration of naive and memory T-cells and their replicative capacity) for each age. These characteristics are used in the second model, the model of infectious disease, to determine the value of the lymphocyte concentration at the beginning of disease and the rate of immune response. This model makes it possible to simulate the course of unified infectious disease for each set of immune characteristics or, in other words, for each age. Disease severity is defined as a maximum of target tissue damage in the course of the disease.

The third model is a function of the distribution of the resistance to infections in the population. Infection resistance is defined as a probability of recovery at a certain value of target tissue damage (disease severity). As an output of the model we have risk assessment of lethal outcome in the course of the disease. To estimate the probability of death from certain diseases during a time interval (e.g., during 1 year) we multiply the risk of lethal outcome in the course of the disease by the probability of becoming infected during the age interval under consideration.

5.1 Relationship Between Disease Severity and the Risk of Death

We define the infection resistance *Res* as a probability of recovery from the disease having the severity value *S*. Then, the probability of the lethal outcome is $p_L = 1 - Res$. Further, we assume that this characteristic is normally distributed in the population. Hence, the probability of the lethal outcome p_L at the severity value *S* could be represented as the corresponding distribution function

The values of the parameter were estimated based on the clinical observations. Thus, by means of the model of infectious disease and expression (3), a relationship between age-related changes in the T-cells population and the risk of death could be established.

$$p_L(S) = \Phi(S) = \int_0^S \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(t-a)^2}{2\sigma^2}} dt \tag{3}$$

5.2 Results of Simulation

The WHO data on pneumonia mortality in Austria, Italy, Portugal, the United Kingdom, the USA, and Japan in 1999 are represented by symbols in Fig. 9. The prob-

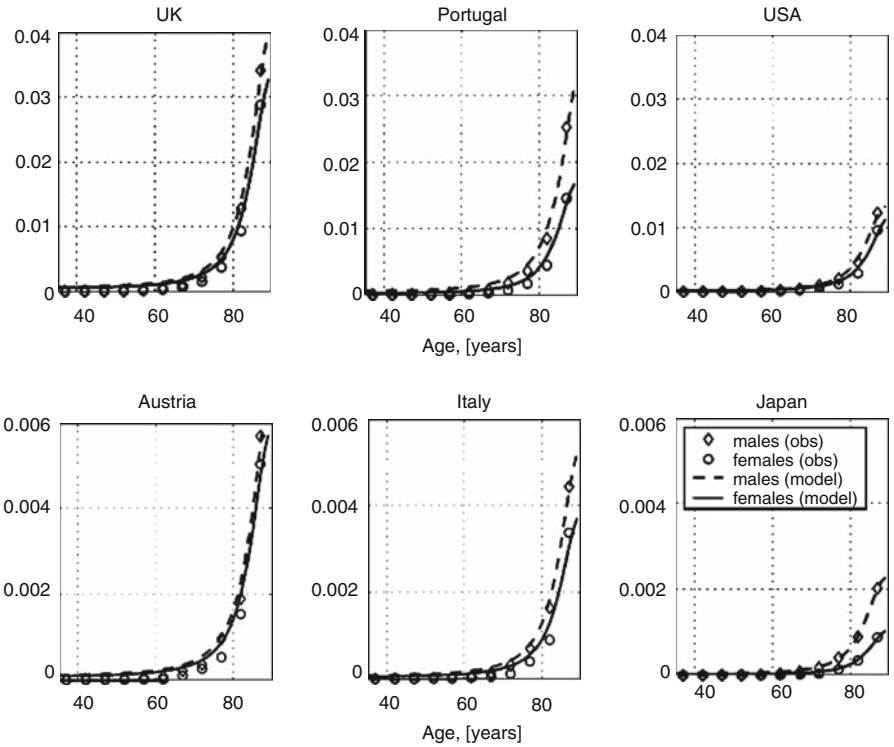

Fig. 9 Pneumonia mortality (probability of death from pneumonia per year) in Austria, Italy, Portugal, United Kingdom, USA, and Japan in 1999. WHO data are represented by symbols, results of simulation by lines

ability of death from pneumonia in the age group 80–84 in the UK is 27 times higher than in Japan and 10 times higher than in Italy.

We assume that these populations experience different antigenic load throughout adult life. This can be related to differences in climatic and ecological conditions, modes of living, and national cuisines. We fit the model of age-related risk of death from respiratory infections to the data. The results of the simulations are represented by the solid and dashed lines in Fig. 9. There is good agreement between the model and the data sets for medium and large values of the death rate. For small values (age group 35–39), the estimated risk of death is higher than observed.

To provide a good fit, two parameters of the model were estimated for every population: the value of the antigenic load and the frequency of pneumonia (Fig. 10). The differences in age-specific mortality between countries are mainly described by variations in the frequency of pneumonia. Males in Japan and in the US have higher estimate of the antigenic load than in other countries under consideration. The higher rate of immunosenescence in the male populations of these countries

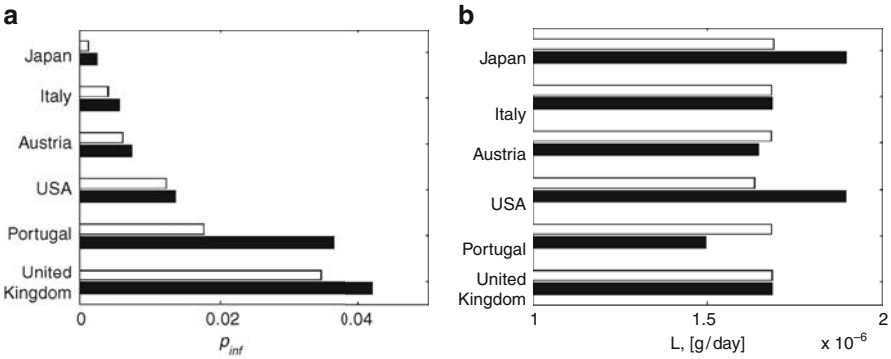

Fig. 10 Parameter estimates of environmental conditions which influenced immunosenescence in the populations under consideration. (a) frequency of pneumonia and (b) antigenic load. Black bars correspond to males; white bars to females

may also be related to the dynamic and stressful mode of living (Epel et al. 2004; Segerstrom, Miller 2004).

6 Conclusion

The proposed model describes the relation between immunosenescence and demographic aging. The initial values of variables in the model (1) correspond to the population average. In the case of availability of the clinical measurements, the proposed model can be transformed into the individualized risk model, which makes it possible to predict consequences of individual interventions. There is growing body of evidence that modification of the immune state by vaccination, antiviral and hormonal therapies, stem cell transplantation, and, possibly, by regulation of telomerase activity (Bodnar 1998), could slow down processes associated with immunosenescence. Mathematical modeling is a convenient tool for testing such intervention strategies.

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Part II
Cellular Immunosenescene - T Cells

Age, T-cell Homeostasis, and T-cell Diversity in Humans

David L. Lamar, Cornelia M. Weyand and Jörg J. Goronzy

Contents

1 Introduction

A fundamental feature of mammalian adaptive immunity is the highly diverse pool of antigen receptors found on lymphocytes. The T-cell receptor and the surface immunoglobulin on B cells facilitate the recognition of foreign structures found on tumors and pathogens that have overwhelmed the defenses of the innate immune system. Because pathogen encounters and neoplasic transformations are inherently unpredictable, an immense lymphocyte receptor repertoire is required to meet all of the possible challenges an organism will face. In young humans, the daily production of naïve B cells from the bone marrow and T cells from the thymus steadily

J. J. Goronzy (✉) · C. M. Weyand · D. L. Lamar
The Kathleen B. and Mason I. Lowance Center for Human Immunology
Department of Medicine
Emory University School of Medicine
Room 1003 Woodruff Memorial Research Building
101 Woodruff Circle, Atlanta, GA
Tel.: (404) 727-7310
Fax: (404) 727-7371
E-mail: jgoronz@emory.edu

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injects the lymphocyte pool with new antigen receptors. Unfortunately, as humans age functional thymic tissue gradually involutes and is replaced by fat. In parallel, the daily production of new naïve T cells declines such that no meaningful thymic T-cell production occurs after the age of fifty. Thus, the T-cell repertoire of an adult human must be maintained for decades in the absence of a replenishing source. Although homeostatic mechanisms are remarkably successful at maintaining the T-cell repertoire for many years, obvious changes begin to emerge with advanced age. Most strikingly, the naïve CD4 T cells that remain after the age of 65 undergo a sudden and dramatic collapse of T-cell receptor diversity. Naïve CD8 T cells may experience an earlier and more gradual diversity loss, although direct evidence for this is not yet available. A steadily expanding memory population maintains total T-cell numbers despite the decline in naïve T cells. Among these memory cells, an increasing percentage acquires a terminally differentiated phenotype characterized by abnormal expression of regulatory receptors and resistance to apoptosis. Oligoclonal populations accumulate after a lifetime of repeated challenges such as chronic infections, leading to a contracted memory repertoire. Although the consequences of repertoire contraction are not yet known, this phenomenon may have important implications for the health of the ever growing elderly population.

This review will first delineate the developmental steps that lead to a diverse naïve T-cell repertoire followed by a discussion of the homeostatic mechanisms required to maintain T cells in the periphery after thymic involution. Some of the techniques employed to monitor thymic decline, peripheral homeostasis, and repertoire integrity will be highlighted throughout. Finally, the impact of aging on maintaining a diverse repertoire with stable representation of functional T-cell subsets will be discussed.

2 T-cell Generation

2.1 T-cell Progenitors

Being highly dynamic and in constant turnover, the T-cell system is dependent on the generation of new T cells. T cells derive from self-renewing, pluripotent hematopoietic stem cells (HSC), the ancestors of all blood cells. Early lineage commitments occur in the bone marrow, but final T-cell differentiation and generation of T-cell diversity is entirely dependent on a functional thymus. HSC first develop into multipotent progenitors capable of becoming both myeloid and lymphoid cells. Additional differentiation leads to the common lymphoid progenitor cells which can become T cells, B cells, and NK cells. Little is known about the exact T-cell progenitor that exits the bone marrow and is destined to enter the thymus [1]. CD34, a marker of HSC in the bone marrow, is expressed on circulating cells with strong in vitro T-cell potential [2]. Intrathymic multipotent precursors also initially express CD34 suggesting that T-cell precursors come from the circulating $CD34^+$ population [3]. Within the thymus, committed T-cell precursors are thought to differentiate

from $CD34^+$ cells that have acquired CD1a expression [4]. Additionally, expression of the Notch1 receptor and signaling apparatus, which are required for T- versus B-cell lineage commitment, are likely characteristics of intrathymic T-cell precursors [5]. Whatever the true characteristics of circulating and intrathymic T-cell progenitors are, T-cell generation is dependent on a continual supply of potential thymocytes entering the thymus for further maturation.

2.2 Generation of T-cell Receptor Diversity

The effectiveness of the human adaptive immune system requires a diverse array of antigen receptors on lymphocytes. In both B and T cells, this diversity arises from the somatic rearrangement of gene segments encoding each subunit of the antigen receptor and the combination of these uniquely encoded subunits to make a complete receptor. For the T cell, this process occurs in the thymus and results in the expression of a single T-cell receptor (TCR) consisting of two rearranged receptor subunits. The vast majority of T-cells express receptor chains encoded by the TCRA and TCRB loci and are called αβ T-cells. The remaining T-cells (2–14% of peripheral T-cells [6]) are called γδ T cells and have TCR encoded by the TCRG and TCRD loci. This discussion will focus only on αβ T cells.

To appreciate what T-cell diversity generation entails, the remainder of this section will detail the intrathymic events that transform genetically homogeneous and undifferentiated thymocytes into mature naïve CD4 and CD8 T cells displaying genetically and structurally diverse TCR. The mature TCR is a heterodimer composed of two Type-I membrane-spanning subunits called the α- and β-chains [7]. Each chain has two Ig-like domains and a short transmembrane region. The membrane-proximal Ig-like domains are called constant domains because they are nearly identical on all αβ T cells. The membrane-distal Ig-like domains differ from T cell to T cell and are called variable domains. They are responsible for antigen recognition and are the basis for the immense diversity necessary to respond to the full array of potential pathogens encountered by naïve T cells.

A schematic representation of TCR genes is depicted in Fig. 1. Early thymocytes do not express any TCR, and both alleles of each TCR gene are fully intact. These $CD34^+CD1a^+$ thymocytes also lack the TCR coreceptors CD4 and CD8 and are thus known as double negative thymocytes. In humans, unlike in mice, before TCR rearrangement begins, thymocytes will express CD4 alone or both CD4 and CD8 in some cases [8]. The first step towards expression of a functional TCR is expression of RAG1 and RAG2 by thymocytes [9]. These proteins are essential for somatic recombination, and once they are expressed, the TCRB locus begins to rearrange. The diversity (D_β) and joining (J_β) gene segments are the first to be combined, followed by joining of a variable (V_β) segment to the D_β-J_β junction. The particular segments included are stochastically chosen and the order of segment joining is dictated by recombination signal sequences flanking the gene segments (reviewed in [10]). During recombination, the enzyme terminal deoxyribonucleotidyl trans-

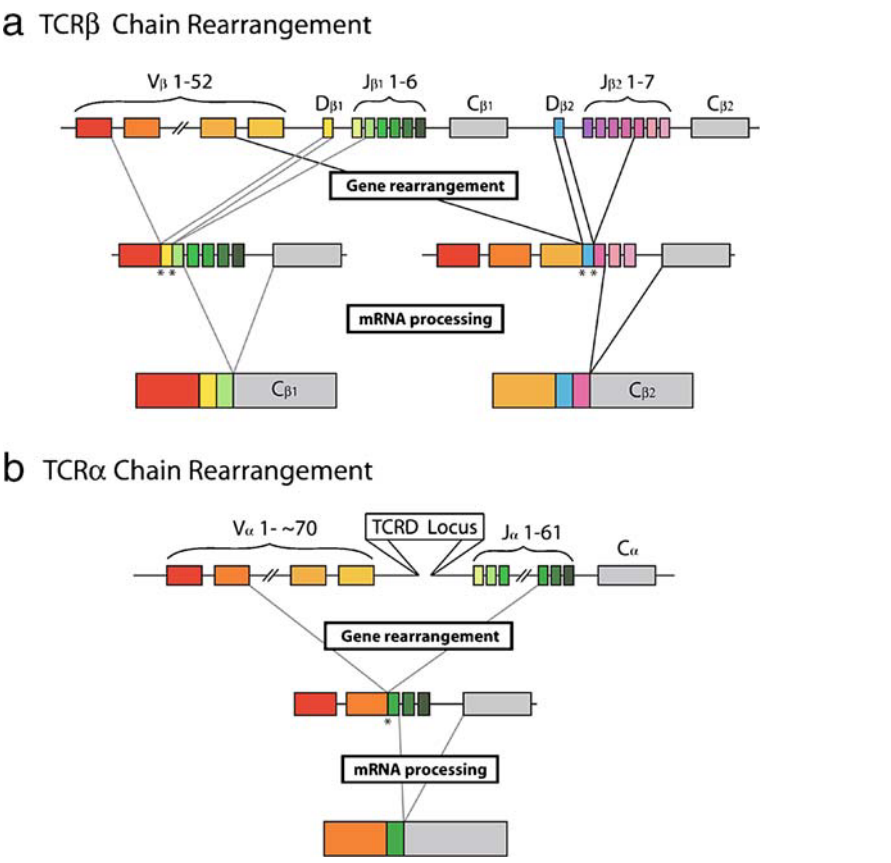

Fig. 1 Rearrangement of TCR Loci in αβ T cells Schematic representations of TCR gene rearrangement and mRNA processing to yield mature TCR chains (gene segments are not to scale). (a) The TCRB locus on chromosome 7 rearranges first. (b) The TCRA locus on chromosome 14 rearranges after successful β-chain rearrangement and expression. The TCRD locus lies within the TCRA locus and is deleted when TCRA rearrangement occurs. * indicates sites of terminal deoxyribonucleotidyl transferase-mediated generation of junctional diversity

ferase (TdT) facilitates the random addition and subtraction of a variable number of nucleotides at segment junctions [11]. This imprecise joining, termed junctional diversity, is an important source of variable domain sequence diversity for both TCR chains. As depicted in Fig. 1, the TCRB locus has two C_{β} gene segments. A failed attempt to rearrange the locus using the $C_{\beta 1}$ gene segment can be followed by an attempt to use the $C_{\beta 2}$ locus on the same chromosome. When the TCRB locus is successfully rearranged, the resulting protein will be expressed at the surface in complex with the pre-Tα-chain, an invariant surrogate required for β-chain expression [12]. Expression of this pre-TCR signals completion of β-chain rearrangement. At this time, RAG1/2 expression ceases, preventing further rearrangement of the second TCRB allele and ensuring that all T cells express a single β-chain [4].

An additional consequence of successful TCRB rearrangement and pre-TCR expression is a massive proliferation of β-chain$^+$ thymocytes before TCRA rearrangement. By expanding at this stage, each unique successful β-chain rearrangement can be combined with many different α-chains, thereby dramatically enhancing the total TCR repertoire. It is estimated that roughly 10 divisions occur between β-chain expression and TCRA rearrangement, resulting in ~1000 thymocytes with the same β-chain [13]. This number is much higher than the number of distinct α-chains paired with a single β-chain in mature peripheral T cells because only 10% percent of β-chain$^+$ thymocytes will successfully rearrange the TCRA locus and survive positive and negative selection. After expansion, β-chain$^+$ thymocytes reexpress the RAG proteins and TCRA gene rearrangement begins [9]. TCRB and TCRA gene rearrangements proceed by the same mechanism with a few distinctions. Whereas the β-chain variable domains are comprised of V, D, and J segments, the TCRA locus only has V and J segments. Unlike β-chain rearrangement, a cell without a successful α-chain rearrangement can continue rearranging by joining upstream V_α segments to downstream J_α segments until a competent chain is formed [14]. Additionally, surface expression of a rearranged α-chain, in complex with the β-chain, does not silence RAG1/2 expression. Instead, RAG1/2 expression ceases after positive selection (discussed below), which signals completion of TCR gene rearrangement [15]. A consequence of prolonged RAG1/2 expression during TCRA rearrangement is the possible coexpression of more than one α-chain on each T-cell [14]. It is likely, however, that only TCR containing the positively selected α-chain allele will interact with MHC-peptide complexes in the periphery during an immune response.

2.3 Positive Selection and Negative Selection

Thymocytes with successfully rearranged TCR will only emerge from the thymus as CD4 or CD8 naïve T cells if they survive positive and negative selection. These selection processes require interactions between the TCR on thymocytes and the MHC class I and II molecules on bone marrow-derived cells and specialized epithelial cells within the thymus. Because a functional T-cell response requires recognition of pathogenic peptides presented in complex with MHC molecules, only TCR capable of making stable contacts with MHC are useful additions to the T-cell repertoire. Thymocytes must receive signals through the TCR to avoid death by neglect (positive selection), which is the fate of about 90% of all TCR$^+$ thymocytes [16]. The requirement for positive selection ensures the elimination of TCR least likely to contribute to an immune response. Additionally, an excessively high affinity interaction will lead to the death of the T cell and removal of that cell's TCR from the repertoire (negative selection). Because only self-peptides are presented on MHC molecules in the thymus, negative selection removes T cells with potentially autoreactive TCR. About half of the remaining 10% of TCR$^+$ thymocytes are removed by negative selection [16]. Survivors of both positive and negative selection exit

the thymus as mature T cells expressing a TCR that may recognize an epitope from potential pathogens.

In addition to ensuring that a T cell expresses a fully functional yet self-tolerant TCR, intrathymic TCR:MHC interactions dictate a key determinant of each thymocyte's future function: the choice of CD4 versus CD8 coreceptor expression [16]. A mature T cell whose TCR interacts with MHC class I molecules during positive selection will express CD8. Alternatively, if positive selection occurs against MHC class II molecules, the resulting mature T cell will express CD4.

2.4 How Diverse is the Human T-cell Repertoire?

As stated above, all receptor diversity in the T-cell pool is generated by somatic gene rearrangement during thymocyte maturation. By examining the key sources of this diversity, a theoretical upper limit of distinct αβ TCR has been estimated [7]. Three main factors contribute to the diversity of the TCR repertoire: the inclusion of a single V, D, and J gene segment (V and J only for the α-chain) in the variable domain of each TCR chain, the random addition and subtraction of nucleotides at the junction of combined gene segments (junctional diversity), and the pairing of one rearranged α-chain with one rearranged β-chain to yield a complete TCR. There are ~70 V_α and 61 J_α gene segments which when combined would yield 4,270 different α-chains if all combinations are productive. The 52 V_β, 2 D_β, and 13 J_β gene segments could potentially combine to form 1,352 different β-chains. Without accounting for junctional diversity, 5.8 million different TCR could be generated by combining one α-chain with one β-chain. Estimates suggest that junctional diversity may increase TCR diversity by a factor of 2×10^{11} to a total of 10^{18} potential unique TCR. This estimate is an overstatement of the true repertoire potential because many V_α-J_α and V_β-D_β-J_β combinations result in nonsense frame shifts and not all α-β-chain combinations can be expressed. Even with a more modest estimate, it is clear that the receptor repertoire of any individual, whose total T-cell compartment contains only ~3×10^{11} cells, represents only a minute fraction of the potential diversity.

A variety of techniques used to measure diversity (discussed below) estimate that the T-cell repertoire consists of about 10^8 different TCR in young humans. If TCR were all equally represented, the clonal size of T cells bearing the same receptor would be about 1,000. In reality, certain clones are more abundant than others, and the receptor repertoire differs among the different functional T-cell subsets. Naïve cells, which represent about 50% of total CD4 and CD8 T cells [17–19] in young humans, harbor the majority of total TCR diversity. An assessment of TCR repertoire within CD4 T cells in young donors revealed that memory cells have only 5–10% of the β-chain diversity of naïve cells [19]. As, each β-chain in the memory population is generally paired with a single α-chain, this translates to only 1% of the total naïve αβ TCR diversity.

Because an organism cannot predict which pathogens will be encountered by the immune system, it is beneficial that naïve lymphocyte antigen receptors are highly

diverse. Naturally, the receptor repertoire in the memory compartment can only be, and is only required to be, as diverse as the epitopes against which a primary response has already been raised. However, in addition to receptor diversity, memory T cells comprise several different functional populations. The paradigm of the memory T-cell life-cycle dictates that the cells remaining after the contraction phase of a primary immune response will be either central memory or effector memory cells [20]. Central memory cells, defined as $CD45RA^-$ and $CCR7^+$, reside in lymphoid tissues such as the spleen and lymph nodes. Effector memory cells, defined as $CD45RA^-$ and $CCR7^-$, are found in peripheral sites where antigen exposure is most likely, such as the skin, lungs, and GI tract.

In addition to distinct homing patterns, central and effector memory T cells respond differently upon antigen reexposure [21]. While both memory subsets are much more sensitive to TCR stimulation than naïve T cells, effector memory cells have an even lower activation threshold. Consistent with their localization at the frontlines of antigen exposure, effector memory cells are quickly and potently triggered to produce effector cytokines such as IFN-γ, a key molecule in a strong immune response. Conversely, central memory cells likely reencounter antigen only after it has been delivered to lymphoid tissue and presented on antigen-presenting cells. After stimulation, these cells produce high amounts of IL-2 which promote expansion of activated T cells. Several studies in mice suggest that central memory cells differentiate into effector memory cells, thereby enhancing the current immune response and seeding the periphery for future exposures [22, 23]. A third memory subset, the terminally differentiated effector cell (known as CD45RA effector cells), is defined by reversion of effector memory cells to CD45RA positivity while still lacking CCR7 [24]. These cells are much more common in the CD8 compartment and are characterized by potent cytotoxicity, resistance to apoptosis, and weak proliferative potential. As we will discuss later, CD45RA effector cells accumulate with age and may result in altered functionality of memory responses in the elderly.

2.5 Techniques for Assessing Diversity

Before discussing the mechanism of diversity maintenance and the TCR repertoire changes that occur with age, we will discuss the techniques used to estimate human TCR diversity. The staggering array of unique TCR gene rearrangements in peripheral T cells makes direct measurement of diversity a challenge. Only gross changes in repertoire, such as large clonal expansions or the loss of entire V_β families, can be detected using the following low sensitivity methods. Flow cytometry allows detection of differences in V_β family usage among various T-cell compartments or age ranges [25]. However, even a severe loss of repertoire diversity that affected the different V_β families similarly would be missed by this method. TCR clonotyping, which utilizes V_β-C_β or V_α-C_α specific PCR and denaturing gradient gel electrophoresis, relies on the existence of clonal expansions large enough to dominate the PCR products of entire V_β families [26]. Similarly, the "immunoscope" method (see

below) uses PCR to detect large expansions within V_β families [27]. While this technique is only slightly more sensitive than clonotyping, immunoscoping is a useful tool to estimate total T-cell diversity when combined with sequencing [28].

More direct assessments of TCR repertoire all use a common approach: isolate and characterize a small subset of sequences and extrapolate their frequencies, based on parameters such as V_β frequency and possible αβ combinations, to the whole T-cell pool. Wagner et al. examined repertoire diversity using TCR-specific probes and limiting dilution of CD4 T cells [29]. Using primers specific for two V_β-J_β combinations (V_β8-J_β1S4 and V_β18-J_β2S5), representative samples of β-chain gene sequences from CD4 T cells of several donors were obtained. Specific biotinylated probes complementary to the TCR N-D-N region (this region is highly variable and includes the junctional nucleotides that flank the D gene segment) were then generated. In a subsequent step, a second sample of CD4 T cells from the same donor was screened for the presence of these sequences in a limiting dilution system. cDNA was isolated from replicates of serially diluted T cells ranging from 10^5 to 5×10^6 cells, amplified by PCR with the appropriate V_β-J_β primer set and hybridized with the labeled probes specific for the isolated TCR sequences. This method determines the frequency of each specific β-chain sequence in the entire T-cell pool and allows for an estimate of the total diversity. Studies using this method [19, 29] have estimated that a given β-chain obtained from naïve CD4 T cells is present with a median frequency of <1 in 2×10^7 T cells in healthy young and middle-aged adult humans, i.e., the human naïve CD4 T-cell compartment encompasses around 20 million different TCR β-chains. Because the sensitivity of the limiting dilution system is less than 100%, this estimate represents the upper range.

The "immunoscope" technique, mentioned above as a method to detect T-cell clonal expansions [27], has been exploited to estimate human αβ TCR diversity. As with the limiting dilution assay of Wagner, et al. [29], this technique requires PCR amplification across the β-chain N-D-N region followed by sequencing. Previously, it was observed that the lengths of rearranged TCRB transcripts between constant sequences flanking the N-D-N region follow a Gaussian distribution. The span of sizes results from the stochastic addition and subtraction of nucleotides during the joining of gene segments [11]. Electrophoretic separation of PCR products amplified from a heterogeneous T-cell cDNA pool reveals a laddering of discrete bands separated by a gel distance corresponding to 3 nucleotides/1 codon. A graphical depiction of intensities of these bands reveals 6–8 peaks, with the most intense central peak corresponding to an N-D-N region length of 8–10 amino acids. A non-Gaussian spectrum results when one or a few TCR clones are overrepresented.

It has been shown that, when normally distributed, the intensity of each peak is proportional to the diversity of specific TCR sequences present in the peak. To estimate total human β-chain diversity, Arstila, et al. isolated a single band from the separation of V_β18-J_β1.4 PCR products and identified all TCR variants by sequencing [28]. The total number of V_β18–J_β1.4 sequences was extrapolated based on the intensity of the sequenced band and total β-chain diversity was extrapolated from the frequency of V_β18 and J_β1.4 positive T cells in the individuals used for the study. After repeating this procedure for several donors and other V_β-J_β segments, the authors arrived at a minimal estimate of 1.3×10^6 different TCR β-chains.

2.6 Thymic Decline with Age

The anatomy of the thymus is expressly suited to allow for the maturation of new T cells with a staggering array of unique TCR. The thymus is the sole organ where naïve T cells are produced and diversity can be generated or refreshed. Consequently, thymic integrity plays a key role in T-cell repertoire maintenance over the many decades of a human life. In the following section, we will discuss how aging affects the thymus and its ability to provide a steady supply of new naïve T cells and, therefore, new TCR clonotypes.

The thymic architecture consists of the thymic epithelial space and the perivascular space (reviewed in [30]). Thymopoiesis occurs entirely within the thymic epithelial space which includes the cortical and medullary epithelial cells and bone marrow-derived antigen-presenting cells required for positive and negative selection. The perivascular space is located within the thymic capsule and is separated from the thymic epithelial space by a basement membrane. The cellular component of the perivascular space consists of fibroblasts, lymphoid cells, and a few adipocytes and is notable for the absence of thymocytes. The classical description of thymic anatomy, consisting mostly of thymic epithelial space with only a small contribution from perivascular space, applies only to very young human thymi. As early as age 2, the thymic epithelial space begins to decline with a compensatory enlargement of the perivascular space by adipocytosis. Although total thymic mass remains constant, the loss of thymic epithelial space results in a steady decrease of new T-cell production after adolescence.

Numerous studies have observed decreased thymopoiesis with age. The most common techniques for evaluating thymic output involve quantifying the number of recent thymic emigrants in the peripheral blood. In the absence of a reliable surface marker for recent thymic emigrants, many groups have resorted to the detection of TCR excision circles (TREC) which are DNA remnants of TCR gene rearrangements. During thymocyte maturation, chromosomal DNA within the TCR loci is broken and religated to join V or D gene segments with J gene segments and V segments with D-J gene segments. In each case, the DNA sequence between the joined gene segments is excised from the chromosome and the cleaved ends of the deleted sequence are ligated together resulting in a TREC. The correlation of TREC with thymic output follows from the fact that, with each cell division, chromosomal DNA is replicated while TREC DNA is not [31]. After excision of a TREC during TCR gene rearrangement, subsequent mitotic events will dilute the TREC/cell ratio because only a single descendent of the original thymocyte, regardless of the number of future divisions, will retain the TREC. The stability of TREC in the absence of division has been debated, and the persisting TREC with aging may therefore derive from nondividing cells rather than from recent thymic emigrants.

Each TREC represents a single gene rearrangement event, and each step in the TCR rearrangement process can lead to the formation of a different TREC. TREC resulting from the V, D, and J segment-joining events are unique to the particular segments joined and are, therefore, rare in the total population and not useful for a global assessment of recent thymic emigrants. A fortuitous requirement for the

successful rearrangement of the TCRA locus is the deletion of the TCRD locus which is found between the TCRA V and J segments (see Fig. 1b). TCRD deletion requires the joining of two genetic elements flanking the delta locus, δRec and ψJα [32]. The resulting TREC (sjTREC) contains a δRec-ψJα signal joint that is identical for all TCRD deletion events. When the TCRA gene is subsequently rearranged, the δRec-ψJα coding joint will be included in the resulting TREC (cjTREC) regardless of which TCRA segments are joined (diagrammed in [33]). Although either of these TREC can be used for estimating αβ T-cell production, the sjTREC is the more common choice because the δRec-ψJα coding joint can still remain on the incompletely rearranged chromosome in 5% of αβ T cells [33]. An additional advantage of TCRD TREC, which are produced late in TCR rearrangement, is that only 3–4 divisions occur between TCRD deletion and full TCR rearrangement [34]. Thus, δRec-ψJα TREC are minimally diluted in newly matured αβ T cells.

Regardless of which TREC is used to assess recent thymic emigrants, thymic function comparisons can be made among donors of various ages. Multiple studies suggest that thymic production steadily declines with age [34–36]. This decline occurs at the rate of about 3% per year, which is the same rate estimated for the loss of thymic epithelial space with age [37, 38]. TCR rearrangement in thymocytes occurs normally regardless of age, generating a diverse Vβ repertoire [39]. Additionally, newly generated T cells in individuals up to at least age 50 perform as well as young T cells in in vitro assays. Therefore, it is thought that while total thymic epithelial space and consequently new naïve T-cell production declines with age, T-cell production in the remaining tissue is qualitatively intact [30]. Quantitatively, however, the thymus is unable to provide a meaningful supply of new naïve T cells after middle age as evidenced by the small numbers of peripheral TREC. Additionally, the ability to reconstitute the T-cell compartment following ablative bone marrow transplantation decreases steadily with age. In fact, patients in their fifties fail to return to pretreatment cell numbers even 2 years after transplant [40].

TREC levels in T cells are used as an indirect surrogate of thymic T-cell output and many papers treat $TREC^+$ naïve T cells as recent thymic emigrants. However, it has been noted that the interpretation of TREC measurements is more complicated [33]. Indeed, decreased thymic output results in dilution of the TREC/cell ratio over time; so does homeostatic proliferation or death of existing $TREC^+$ T cells. For this reason, TREC measurements tend to overestimate thymic output and interpretations should take into account the kinetics of T-cell turnover. Recently, one group has proposed a new marker for recent thymic emigrants that may help resolve the potential ambiguity of TREC dilution. Kimmig et al. reported that CD31 positivity on $CD45RA^+RO^-$ naïve T cells correlated strongly with the presence of sjTREC [41]. Conversely, $CD45RA^+RO^-CD31^-$ T cells, although functionally and phenotypically naïve, have almost no TREC. The authors suggest that recent thymic emigrants lose CD31 expression upon antigen-induced or homeostatic proliferation, an idea that is supported by the loss of CD31 by in vitro culture. An examination of the percentage of CD4 T cells with a $CD45RA^+CD45RO^-CD31^+$ phenotype with age revealed a steady decline similar to that seen when TREC are examined. It remains to be seen whether CD31 will become a widely used marker for recent thymic emigrants, but

the results of Kimmig and colleagues lend credence to the TREC-based findings that thymic production of new naïve T cells declines with age.

3 Maintenance of Diversity

In the absence of foreign antigen, all peripheral T-cell pools experience a steady-state turnover characterized by cell loss (to attrition, death, or phenotypic shift) and compensatory proliferation. In order to maintain the original diversity of recent thymic emigrants, death and replacement of naïve T cells must be completely random so as not to preferentially deplete or replace T cells of certain specificities. An additional requirement for maintaining diversity is that each clonal population expressing a given TCR is of an adequate size to ensure that normal cell death will not eliminate a TCR from the repertoire. The latter requirement is met by proliferation of thymocytes bearing a functionally rearranged TCR before and shortly after mature naïve T cells exit the thymus. Fully random steady-state turnover of peripheral T cells is much more difficult to achieve. In the context of thymic export of new naïve T cells, this problem is not likely to result in a compromised repertoire. However, the severe and early decline of thymic production of T cells seen in humans suggests that peripheral homeostatic mechanisms are crucial for life-long TCR diversity.

Maintenance of the TCR repertoire is a balance between factors that introduce or preserve diversity and those that pose a threat (see Fig. 5). Naïve and memory T cells share some of these factors. For both cell types, the thymus is the ultimate source of new diversity; however, the proximal source of diversity in the memory compartment is activation and differentiation of naïve cells in response to antigen. Therefore, establishment of the memory repertoire depends both on the initial naïve repertoire and the history of antigen exposure. T-cell activation against a novel antigen, while seeding the memory compartment after contraction of the primary response, represents an important threat to naïve T-cell diversity. Normal daily turnover leads to loss of T cells, and potentially TCR, from all compartments. Assuming an adequate initial clonal size, homeostatic proliferation of remaining T cells will replace lost cells and maintain normal compartment sizes. While total T-cell numbers can easily be maintained by replacement proliferation, it is unlikely that all TCR clones are lost and replaced with equal kinetics. Consequently, over many years of homeostatic maintenance, the TCR representation, for both memory and naïve cells, is at risk of skewing and contraction.

3.1 Assessment of T-cell Turnover

A crucial component to T-cell homeostasis, and therefore maintenance of T-cell heterogeneity, is cell turnover. The persistence of a T-cell clone with a given TCR

depends on proliferation of existing cells in the face decreased thymic output and daily cell loss. It is important to understand the power and the limitations of the techniques used to examine T-cell kinetics and turnover in humans before reviewing the available data.

3.1.1 Ki67 Staining

One easy method for assessing the fraction of proliferating cells within a lymphocyte population is by intracellular staining for Ki67. Ki67 is a nuclear antigen expressed in the G_1, S, G_2, and M phases of proliferating cells. Its absence in resting G_0 cells makes it a good marker of cycling cells [42]. Using standard flow cytometric identification of intracellular antigens [43], a panel of antibodies can be designed to assess the proportion of proliferating cells within T-cell subsets at a given time. This method has been used to estimate the T-cell turnover rates in several contexts, including HIV and aging [19, 44, 45]. The accuracy of Ki67 staining has been confirmed by more advanced metabolic labeling techniques (see below) that simultaneously assess proliferation and loss from a population. While these newer techniques provide a more complete picture of T-cell kinetics, Ki67 staining remains a useful method for immediate ex vivo assessment of proliferation within peripheral blood subsets.

3.1.2 Deuterated Glucose or Water Incorporation

To obtain direct in vivo measures of human T-cell kinetics, several studies have utilized deuterated glucose or water [46–49]. Each of these methods allows for the labeling and monitoring of dividing cells because glucose and water contribute molecules to the biosynthesis of DNA. Consequently, in the presence of these deuterated substances, a certain percentage of molecules in newly synthesized DNA will be labeled with deuterium, ^2H [47, 48]. After a defined administration period, the ^2H source is discontinued and blood samples are taken at several time points to determine the ^2H content in the DNA of cell types of interest. Measurements made before and soon after delivery of labeled glucose or water are used to determine the percentage of cells that proliferate during the administration period. Further measurements made at multiple later time points allow for calculation of the loss rates and half-lives of the isolated cell subsets.

Because deuterated glucose and water are neither radioactive nor a mutagenic threat, they are useful tools for in vivo studies of human T-cell kinetics. The use of ^2H_2O is the more recent of the two labeling methods and, as described by Neese, et al. [48], has financial, feasibility, and experimental advantages over deuterated glucose. For example, deuterated water can simply be added to normal drinking water whereas glucose must be administered intravenously. Without the need for expensive supervised infusion, deuterated water can be delivered over a longer period of time which is beneficial for examining low turnover cell types such as naïve T cells.

3.2 T-cell Homeostatic Mechanisms

In light of the well-documented steady decline in thymic production of new naïve T cells with age, the kinetics and mechanisms of T-cell survival and turnover are vital for maintenance of a diverse and functional adaptive immune system. Using techniques described above, direct estimates of replacement rates and half-lives of human T-cell subsets have been made in vivo. Examination of T-cell kinetics in young adult humans by deuterated glucose [46] revealed that about 0.59 and 0.45% of naïve ($CD45RA^+$) CD4 and CD8 T cells, respectively, proliferate each day. These figures translate to a replacement time of 118 and 145 days, respectively. $CD45RO^+$ memory T cells divide much more frequently than naïve cells. 2.65% of CD4 and 5.09% of CD8 memory T cells proliferate each day, which corresponds to replacement times of 26 and 14 days, respectively. Additional studies by the same group [49, 50] and Ki67 staining of CD4 T-cell subsets by our group [19, 29] confirmed these estimates. Extrapolations from these data suggest that a total of 4×10^9 T cells are produced by peripheral expansion each day [46]. As total T-cell numbers remain stable over time, a comparable loss of T cells also must occur each day. Without a constant supply of new naïve T cells from the thymus, and keeping in mind the requirement of fully random replacement of T cells to maintain a complete TCR repertoire, this extensive daily turnover poses a potential threat to TCR diversity in adults.

3.2.1 Naïve T-cell Homeostasis

In a young, healthy human, the normal daily turnover of naïve T cells is replaced with new T-cell production by the thymus and by proliferation of existing naïve T cells. Our examination of neonatal T-cell kinetics showed that about 10% of daily naïve T-cell production in infants comes from the thymus [45]. Other estimates based on modeling suggest that, at the age of twenty-five, about 20% of the peripheral naïve T-cell pool is populated directly from the thymus [51]. Therefore, even in individuals with an intact thymus, peripheral expansion is the major source of circulating naïve cells. As long as the thymus continues injecting new TCR into the naïve pool, extensive homeostatic proliferation does not threaten receptor diversity. However, as thymic involution proceeds, the burden of maintaining the naïve pool increasingly lies with homeostatic mechanisms. In fact, by age 55, the thymus contributes, at maximum, 5% of the peripheral naïve T cells [51] and probably much less.

Mounting evidence suggests that survival and proliferative maintenance of human T cells in the periphery depend on both TCR- and cytokine-delivered signals. In the absence of exogenous antigen, TCR:MHC interactions in the periphery are analogous to those that mediate positive selection in the thymus; namely, a self-peptide conjugated with the appropriate MHC allele acts as a ligand to provide a survival signal to T cells with a TCR that binds with sufficient affinity. Studies in mice do indeed suggest that peripheral naïve T cells are lost in mice lacking MHC molecules. CD4

T cells are vastly reduced in animals lacking MHC class II [52, 53] and similarly, adoptively transferred CD8 T cells will only persist in mice that express MHC class I [54, 55]. For CD8 T cells, it has been shown that the peripheral MHC requirement extends to the exact class I allele against which the T cell was positively selected in the thymus [54]. Additional studies show that peripheral T cells rely on TCR-generated signals not only for survival but for homeostatic expansion [56, 57].

The role and requirement for a TCR-generated signal to maintain T-cell homeostasis has implications regarding the long-term integrity of the TCR repertoire in the context of dwindling thymic output. If homeostatic responses depend on the strength of the TCR:MHC interaction, it is necessarily the case that some TCR will more readily receive survival or expansion signals. An obvious consequence of this scenario is nonrandom TCR maintenance and/or proliferation and, therefore, repertoire skewing. Because a given TCR's affinity for a self-peptide:MHC complex is unlikely to predict a strong response to a pathogen peptide:MHC complex, the skewed repertoire generated by self-antigen-driven homeostatic proliferation would not only deplete T-cell clonal diversity but would do so without promoting enhanced protection.

Fortunately, antigen-independent stimuli delivered by cytokines also play an important role in peripheral T-cell maintenance. Extensive work in the mouse, and more recently in the human, has established that cytokines signaling via the common γ-chain (which include IL-2, IL-4, IL-7, IL-15, and IL-21) play a key role in peripheral T-cell maintenance and expansion [58]. While the cytokine requirements for homeostasis differ among the various T-cell subsets and with regard to the particular function examined, IL-7 and IL-15 seem to be the most important. In mice, naïve T cells, in addition to TCR:MHC interactions, require IL-7 for steady-state survival in vivo. Although other molecules are likely involved, studies in mice [59] and humans point to upregulation of the antiapoptotic protein Bcl-2 as a mechanism for cytokine-mediated T-cell survival [60].

Homeostatic expansion of peripheral T cells is controlled by the same general mechanisms that mediate survival. Steady-state naïve T-cell turnover, as discussed above, is quite low compared to memory T cells. This implies that memory cells have a stronger proliferative response to the required signals. While this may be true, naïve T cells are capable of a robust expansion in lymphopenic situations [61]. From these studies it can be argued that, while naïve T-cell survival and proliferation require the same TCR- and cytokine-mediated signals, space in the compartment is also necessary for homeostatic expansion. In the steady state, the characteristic naïve and memory T-cell replacement rates [46] may be a direct response to fill compartmental space made available by attrition or cell death.

4 Aging and the Loss of T-cell Heterogeneity

Given that thymic production is the only source of new naïve T cells, and that very little meaningful thymic output remains after early adulthood, one may expect dramatic changes in the peripheral T-cell pool with age. In fact, some obvious changes occur while other features remain intact. Total T-cell numbers decrease only slightly

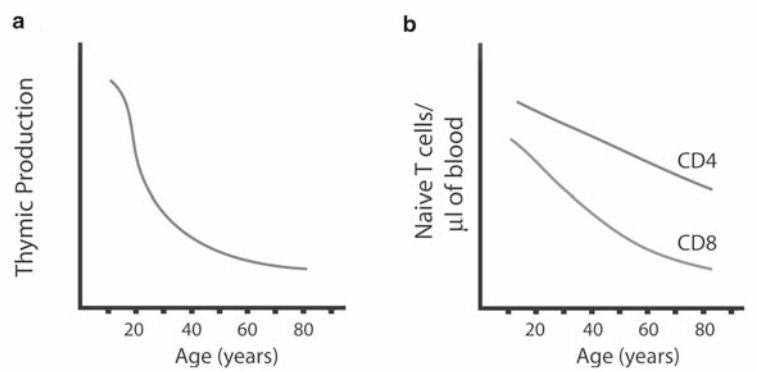

Fig. 2 Naive T-cell Production and Maintenance with Age (a) Schematic representation of loss of thymic production of naive T cells with age. (b) Schematic representation of loss of peripheral naive T cells with age

with age, exhibiting a steady but shallow decline throughout adulthood [17]. Although the maintenance of T-cell numbers is remarkable considering negligible thymic output, the composition of peripheral T cells in the elderly becomes increasingly distinct from young individuals. A consistent and striking finding is the significant loss of naïve cells with age (depicted in Fig. 2). About 50% of young adult T cells are naïve compared to about 35% in 70-year-olds [17]. Remarkably, this naïve loss is much more pronounced for CD8 T cells than for CD4 T cells. In fact, by the age of 70, the percentage of naïve CD8 T cells is consistently around 10%. In contrast, naïve CD4 T cells still make up about 40% of total CD4 T cells [17, 19, 62]. At ages beyond 70, naïve CD4 T cells continue to decline at a steady rate

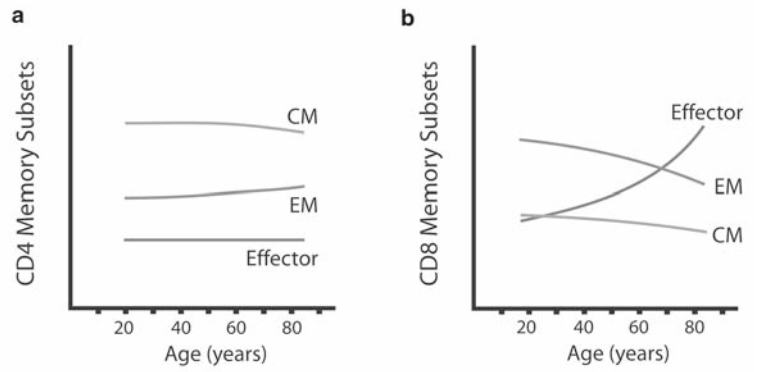

Fig. 3 Changes in Memory T-cell Subsets with Age (a) Schematic representation of changes within CD4 memory T-cell subsets with age. (b) Schematic representation of changes within CD8 memory T-cell subsets with age. CM, central memory; EM, effector memory; Effector, $CD45RA^{+}$ effector memory

whereas naïve CD8 T cells drop to single digit percentages and are virtually gone in individuals who reach 100 [17].

Naturally, with declining naïve T-cell numbers in the context of only slightly reduced total T-cell numbers, the percentage of memory T cells increases with age. In addition, it is clear that memory populations are also qualitatively different in the elderly (depicted in Fig. 3). As appears to be the case for most features of T-cell aging, the CD8 memory T-cell pool experiences a more striking transformation than does the CD4 memory T-cell pool. In young adults, about half of the CD8 memory T-cells have an effector memory phenotype [18, 63]. The remaining half is split equally between central memory and CD45RA effector cells. Throughout adulthood, these proportions shift in favor of CD45RA effector cells which, by the mid-seventies, represent over 50% of all memory CD8 T cells [63]. Because the memory pool as a whole is increased, this translates into more than a 4-fold increase in the absolute numbers of circulating $CD8^+$ CD45RA effector cells. Even central and effector CD8 memory T-cell subsets in the elderly, while comprising a decreased percentage of total CD8 memory T cells, are increased in absolute numbers relative to young adults. Within the CD4 memory T-cell population, aging does not have a significant effect on subset distribution [63]. However, as total memory cells are increased, the absolute numbers of each subset within the CD4 memory T-cell population does increase with age.

A well-established feature of memory T-cell aging is the loss of the costimulatory molecule CD28 [64]. Additional molecules have been identified whose patterns of expression changes with age. Interestingly, many of these molecules, like CD28, are immunoregulatory receptors that might alter the ability of T cells to respond to antigen. Similar to the effects of age on memory T-cell subset distribution, CD8 T cells more readily exhibit these changes than do CD4 T cells. For example, up to 70% of CD8 T cells have lost CD28 by age 80, compared to a maximum of 25% of CD4 cells [63]. Similarly, CD85j, an inhibitory receptor for most classical and nonclassical MHC class I molecules [65], is dramatically increased on memory CD8 T cells in the elderly [63]. This acquisition, which in CD8 T cells is as robust as the loss of CD28, occurs on only a very small subset of CD4 T cells. The distinct behaviors of aging CD4 and CD8 T cells, which are also seen in nonhuman primates [66], extends to more artificial human settings as well; long-term in vitro culture of T cells, which mimics aging in many molecular respects, induces characteristic changes much more readily in CD8 T cells [63].

4.1 TCR Repertoire Loss with Age

In light of thymic decline and the steady decrease in naïve T cells with age, it is important to know whether the naïve TCR repertoire undergoes a similar contraction. Our lab developed a technique (described above) to estimate TCR β-chain frequency in an individual. Two studies of naïve CD4 T cells [19, 29] using this method revealed that, in young individuals, the median frequency of T cells bearing

a given β-chain is <1 in 20 million. This predicts a total β-chain repertoire of 20 million in naïve CD4 T cells. This value is an upper estimate due to the suboptimal sensitivity of the limiting dilution system used. Arstila, et al. used the immunoscope technique, which should underestimate true diversity, to arrive at a figure of 1–2 million different β-chains in naïve CD4 T cells in the young [28]. The actual value is probably in between this and our estimate.

How does the naïve CD4 TCR repertoire change with age? Remarkably, in spite of little to no thymic output, β-chain sequences from individuals up to the age of 65 are present in frequencies similar to those of 20-year-olds. Not only do the most infrequent β-chains represent over 60% of the tested sequences, resulting in the same median frequency of <1 in 20 million, the 60- to 65-year-old donors do not have an increase in overrepresented β-chain sequences; only 20% of tested sequences in both age groups are more frequent than 1 in 1 million. These findings indicate that homeostatic mechanisms effectively maintain the naïve repertoire through age 65 even without significant input of new T cells from the thymus.

When individuals in their late seventies are examined, a dramatic change in naïve CD4 T-cell repertoire maintenance is revealed. Whereas in younger individuals, about 60–70% of β-chain sequences examined are less frequent than 1 in 5 million, nearly 100% of β-chain sequences from 75- to 80-year-old individuals are more frequent than 1 in 1 million. In fact, the majority of sequences are present at a frequency greater than 1 in 200,000. Thus, in a single decade, 99% of the β-chain repertoire is lost. A similarly dramatic repertoire collapse is seen in the memory compartment of CD4 T cells in the elderly (depicted in Fig. 4). This may reflect a generalized break down in T-cell homeostatic mechanisms at advanced age.

While a similar examination of the naïve CD8 T-cell repertoire in the elderly has not been done, indirect evidence suggests that diversity loss occurs earlier and more steadily in CD8 T cells. As discussed above, the loss of naïve CD8 T cells is more severe than for CD4 T cells (see Fig. 2). The corresponding homeostatic pressure to counteract this loss may proceed without preservation of the TCR repertoire. Indeed, CD8 T-cell clonal expansions emerge early in life and continue to accumulate with age [67]. As depicted in Fig. 4b, clonal expansions are much less common and occur later in life with the CD4 T-cell compartment [68]. While many

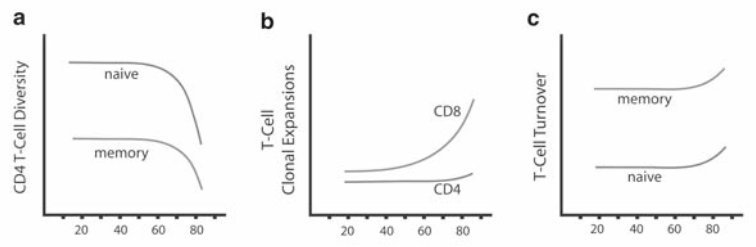

Fig. 4 Changes in T-cell Heterogeneity and Turnover with Age (a) Schematic representation of CD4 TCR repertoire collapse after age 65. (b) Schematic representation of accumulation of CD4 and CD8 T-cell clonal expansions with age. (c) Schematic representation of increased peripheral T-cell turnover late in life

expanded clones come from the memory compartment, naïve TCR clones may each respond differently to increased homeostatic stimuli, and unbalanced proliferation may compromise the diversity of the dwindling naïve compartment. Future studies that directly assess naïve CD8 T-cell diversity are needed to more fully appreciate the consequences of age on the naïve T-cell pool.

In the absence of new thymic T-cell production, naïve CD4 TCR diversity is maintained until the seventh decade of life. Similarly, the naïve CD8 T-cell population, while experiencing a steadier decline in numbers and probably diversity, is remarkably stable for many years after thymic involution. Why, after years of successful repertoire preservation, does this maintenance begin to fail? One possibility is that, with a minimal influx of new T cells from the thymus, peripheral homeostatic mechanisms are capable of maintaining a diverse repertoire [69]. In this case, the collapse of naïve diversity after age [65] may follow the *absolute* end of thymic production, thereby overwhelming homeostatic mechanisms.

Alternatively, evidence from humans and nonhuman primates points to a failure of homeostasis late in life. For both human naïve CD4 T cells [19] and rhesus macaque naïve CD4 and CD8 T cells [70], T-cell turnover significantly increased in old individuals (depicted for humans in Fig. 4c). In macaques, the increases proliferation seems to be a reaction to increased cell loss. In fact, animals with the smallest naïve compartments experience the most dramatic cell turnover. As the authors suggest [70], declining naïve T-cell numbers trigger a compensatory, yet inadequate, proliferation to refill the compartment. This stimulus exacerbates the problem by shifting naïve cells to a memory phenotype, thereby further depleting naïve numbers. Thus, the very mechanism for preserving the naïve population may contribute to its ultimate demise by creating a feedback loop that overwhelms the homeostatic capacity of elderly individuals. The factors leading to declining T-cell diversity in the elderly are depicted in Fig. 5.

5 Implications of Diversity Loss

A wide array of unique TCR within the naïve T-cell compartment is necessary to protect against an unpredictable and diverse antigen pool. Without a naïve response, many ubiquitous and benign infections would be deadly. Emerging neoplastic cells, whose presence would normally be detected by naïve T cells, could survive for the time required to accumulate the additional mutations needed for malignancy. Effective vaccinations require the long-lived memory cells that remain following activation and expansion of naïve T cells specific for the target antigen. Additionally, existing T-cell memory, from past infections or vaccines, must persist to prevent reactivation of chronic infections or reinfection by a familiar pathogen. It seems clear that the elderly, who face a steady decline in naïve T-cells and a dramatic collapse of naïve and memory T-cell repertoire diversity late in life, should respond poorly to challenges requiring full T-cell function. Although infections, cancer, and poor vaccine responses disproportionately affect the very old, it is difficult to assess

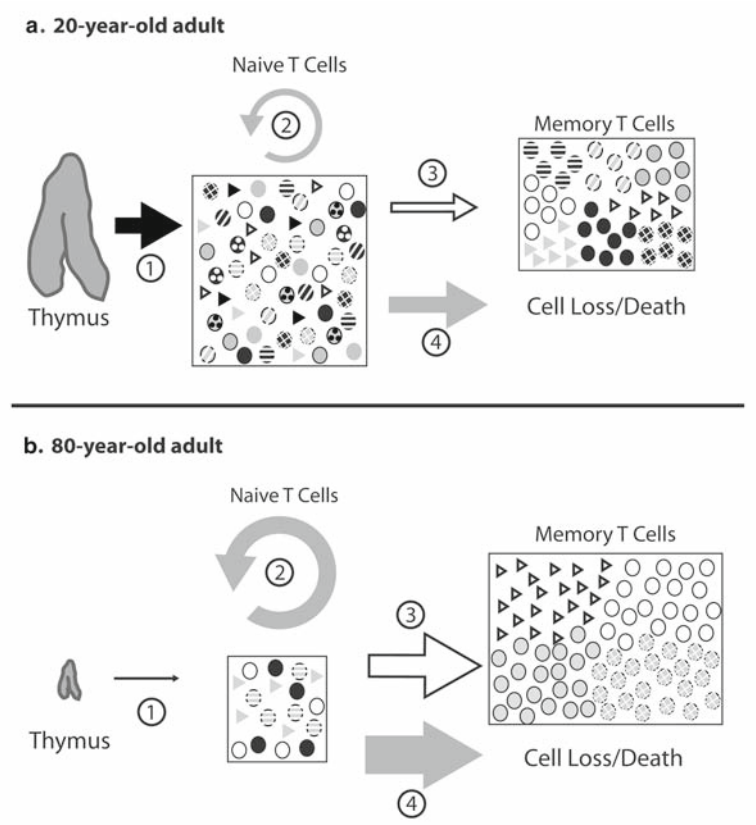

Fig. 5 Determinants of Naive T-cell Diversity (a) Young adult human. 1- A steady supply of new naive T cells continually adds to TCR repertoire. 2- Existing naive T cells proliferate in response to daily cell loss. 3- Exposure to antigen shifts naive clones to the memory compartment. 4- Naive T cells are lost to cell death or attrition. (b) Elderly adult human. 1- Thymus no longer contributes new naive T cells. 2- Declining naive T-cell numbers result in severe homeostatic pressure to proliferate. 3- In addition to antigen exposure, naive T cells shift to memory phenotype because of excessive homeostatic expansion. 4- Naive T-cell death is accelerated due to exhaustion from strong proliferative pressures. Black arrows—promotes diversity of naive TCR repertoire; Black-outlined arrows—depletes naive TCR repertoire; gray arrows—can have neutral and/or detrimental effects on naive TCR repertoire

how much of this is due to a loss of TCR diversity. Several examples of decreased T-cell repertoire in mice and humans hint at the importance of a diverse response.

Many natural and experimentally manipulated genetic backgrounds in mice result in contracted T-cell repertoires. These settings have been used to assess how reduced TCR availability affects immune responses. NZW mice and strains with the *tcr*$^{\alpha}$ haplotype (e.g. C57L) lack certain elements of the TCRB locus [NZW: deletion of $C_{\beta 1}$, $D_{\beta 2}$, and all $J_{\beta 2}$ segments (71); *tcr*$^{\alpha}$: deletion of five V_{β} segments and altered $V_{\beta}10$ sequence (72)] resulting in a 50–60% reduction in potential TCR. T cells

from these mice exhibit reduced ex vivo responsiveness after immunization against some, but not all, tested antigens. TdT-deficient mice are unable to diversify D-J, V-J, and V-DJ junctions during TCR gene rearrangement resulting in a ~90% repertoire contraction. Interestingly, these mice are fully protected from lymphocytic choriomeningitis virus and Sendai virus infections [73]. Although other challenges have not been examined, it is clear that a limited repertoire is capable of enough cross-reactivity to control certain pathogens.

Gene targeting of TCR loci reduces TCR diversity even more dramatically than deletion of the TdT enzyme. For example, all T cells from the TCR OT-1β transgenic mouse express the same TCR β-chain (specific for an OVA peptide when expressed with the OT-1α-chain) and, therefore, all TCR diversity results from TCRA gene rearrangement. In the context of this dramatic repertoire contraction (>98%), these mice are unable to reject allogeneic bone marrow [74]. Other experiments using a different TCR β-chain transgenic strain have shown that some antigen-specific responses are not absent, merely different [75]. After immunization with the bacteriophage protein cl, splenic T cells from both TCR β chain transgenic mice and wild-type littermates responded ex vivo to the cl protein. When challenged with peptides derived from cl, wild-type splenocytes responded most strongly to the peptide known to be immunodominant. In contrast, transgenic mice responded more strongly to a different peptide that yielded no response in wild-type splenocytes. Interestingly, both strains of mice were equally capable of a strong response to both peptides when the peptide, rather than whole cl protein, was the immunizing antigen. Therefore, it seems the severe repertoire contraction in these TCR β-chain transgenic mice created a shift of immunodominance without creating a "hole" in the repertoire. That is to say, T cells from these mice *can* respond well to the classical immunodominant peptide but, when faced with the full protein antigen, expand more readily against a different peptide.

From the above examples, it is clear that the requirement for TCR diversity in the mouse is context dependent. This is most likely true for humans too, although direct examination of repertoire contraction in humans is more difficult. A single patient with a partial X-linked severe combined immunodeficiency (Xid) reversion has provided a rare glimpse at the in vivo consequences of TCR contraction in humans. Although family and clinical history and gene sequencing suggested the patient had Xid, a disease caused by the genetic lack of a functional cytokine common γ-chain [76], he had a nearly full-sized T-cell compartment [77]. The Xid mutation usually blocks T-cell development by preventing IL-2 signaling. In this patient, a compensating somatic mutation in the common γ-chain gene occurred in a single T-cell precursor allowing descendants of this cell to undergo normal thymic development. Immunoscope analysis at age 3 revealed that extensive post-thymic expansion of an estimated 25,000 distinct TCR clones managed to fill the T-cell compartment [78]. In vitro challenge of T cells with a variety of antigens revealed blunted responsiveness. While some in vivo T-cell functions, such as a skin test for the BCG vaccine, were intact, it is unclear if these responses would be protective in the face of a pathogen challenge [78]. Subsequent follow-up has not been reported,

and it remains to be seen if this patient will be at increased risk of immune deficiency in the future.

An in vivo Hepatitis C virus study in chimpanzees revealed a setting where the antigen-specific CD8 T-cell repertoire predicted the outcome of infection [79]. A cohort of animals were all inoculated with an identical strain of virus and followed. Some animals exhibited a diverse array of TCR antigen-binding domains within virus-specific T cells while other responses were more homogeneous. Those animals with a narrow repertoire among responding T cells were more likely to carry viral escape mutants and to never clear the infection. This homogeneity preceded viral escape and persisted after a response was mounted to the new mutants. Presumably, animals with more diverse responses were able to respond to viral mutations as they arose, thus preventing expansion of novel epitopes. These findings highlight the contribution of TCR diversity to a flexible immune response capable of adapting to pathogens that evolve during an infection.

Indirect evidence from studies of human aging suggests that age-related repertoire collapse may indeed have detrimental effects on survival. A longitudinal examination of octogenarians in Sweden revealed an "immune risk phenotype" (IRP) that predicted 2-year mortality. Individuals with the IRP, which was originally defined as inversion of the CD4:CD8 ratio (normal is >1; IRP is <1) and decreased in vitro T-cell proliferation, were more likely to die within the 2-year follow-up period compared to non-IRP individuals [80]. Additionally, the prevalence of the IRP doubled from 16 to 32% over the 2 years. A subsequent study showed that a decreased CD4:CD8 ratio alone is an adequate marker of the IRP and its accompanying increased mortality risk [81]. Importantly, as the proportion of CD8 cells increases, the contribution to total CD8 T cells comes from fewer and fewer clonotypes [26]. Thus, in this elderly population, the individuals with the least diverse CD8 populations were more likely to die during the 2-year study period. While the direct cause of increased mortality is unknown, this correlation is consistent with other studies linking CD8 clonal expansions to decreased EBV [82] and influenza responses [83, 84].

Interestingly, a more recent report following the nonagenarians found that those individuals who reach the age of 100 are free from the IRP and have T cells more similar to middle-aged individuals than even the younger-elderly [85]). For example, the CD4:CD8 ratios of centenarians are not only >1 but are consistently more CD4-heavy than even individuals in their early nineties. Additionally, centenarians had fewer $CD8^{+}CD28^{-}$ T cells, by absolute numbers and as percent of total CD8 T cells, than younger-elderly. As the authors suggest, the collective characteristics of the centenarian population obviously excludes those individuals who have died and, therefore, may represent features of "successful aging." For this reason, a longitudinal examination of T-cell repertoires in the very old may contribute to our understanding of the requirement of TCR diversity for longevity. Can TCR diversity within various T-cell compartments predict mortality as successfully as the IRP? Do the successfully-aged exhibit the dramatic collapse of the naïve CD4 repertoire that we have found in individuals after age 75? Answers to questions like these will help clarify the true in vivo importance of declining TCR diversity with age.

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The Role of T-regulatory Cells in Immune Senescence

Paul Moss

Contents

1 Introduction

T-regulatory cells have come to dominate immunology over the last decade [1]. The ability of cellular components of the immune system to suppress immune function was noticed more than 20 years ago and was recognized by a number of highly cited publications [2]. However, the lack of a specific phenotype for these cells, and an inability to document precise physiological mechanisms for their action, limited their investigation by detailed experimental study. The pioneering work of Sakaguchi and colleagues reestablished regulatory cells and has generated a field of research that extends into all areas of clinical immunology, including immune senescence [3].

At first sight, the concept that the immune system must require some form of cellular control of immune activation is surely no surprise. There are very few physiological systems that can proceed without any form of feedback mechanism and it is now appreciated that there are many different subsets of regulatory cells involved in immune haemostasis. By far the most widely recognized is a CD4+ T-cell population that expresses high levels of CD25. The CD4+CD25+ subset has become the prototypic "T-regulatory" cell in both murine and human studies and has provided the basis for many thousands of publications. However, the fact that CD25 is also expressed on all activated effector T-cells has lead to the quest for a "third marker" that will provide a more definitive and unique phenotype for the regulatory population. Several of these molecules have been suggested over recent years, including

P. Moss (✉)
Institute for Cancer Studies
University of Birmingham, Vincent Drive
Birmingham B15 2TT, UK
E-mail: p.moss@bham.ac.uk

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GITR and low expression of CD127. However, it is the transcription factor FOXP3 that has emerged as the most reliable marker for this functional subset [4, 5] (Fig. 1). FOXP3 is a member of an important family of transcription factors that are involved in processes such as language development and which seem to have a central role in development of developmental functions. This is supported by observations in mice deficient in FOXP3 gene function, which develop an autoimmune condition secondary to unregulated immune responses. Even more compelling are the findings in rare human individuals that were born without FOXP3 function and suffer from an unusual syndrome of autoimmune disease dominated by inflammation of endocrine organs. Although there is some evidence that FOXP3 may be up-regulated on activation of effector T-cells in humans, most authors now agree that this marker is a valid and reliable marker of functional regulatory phenotype across both species. Unfortunately, one concern with the use of FOXP3 is that its detection requires the permeabilisation of cells and therefore renders cells unviable for further downstream analysis. For this reason, a surface membrane phenotype which would allow for the cloning and isolation of regulatory cells would provide a significant boost to this area of research. In 2006 low level expression of CD127 emerged as a good marker of FoxP3 expression in regulatory CD4+ T-cells [6, 7]. CD39 is the most recently evaluated phenotypic marker and is positively associated with FoxP3 expression [8].

The mechanism of action of T-regulatory cells remains the subject of active investigation and it is perhaps fair to say that no consensus has emerged on this topic. Some regulatory populations appear to secrete immunosuppressive cytokines such as IL10 or TGFβ but these properties are not documented in all subsets. Cytotoxic activity has also being observed in some experiments. The target cells for T-regulatory function are also uncertain, as it appears that regulatory cells can act both at the level of antigen presenting cells, and therefore modulate induction of immune responses, as well as act directly on effector cells.

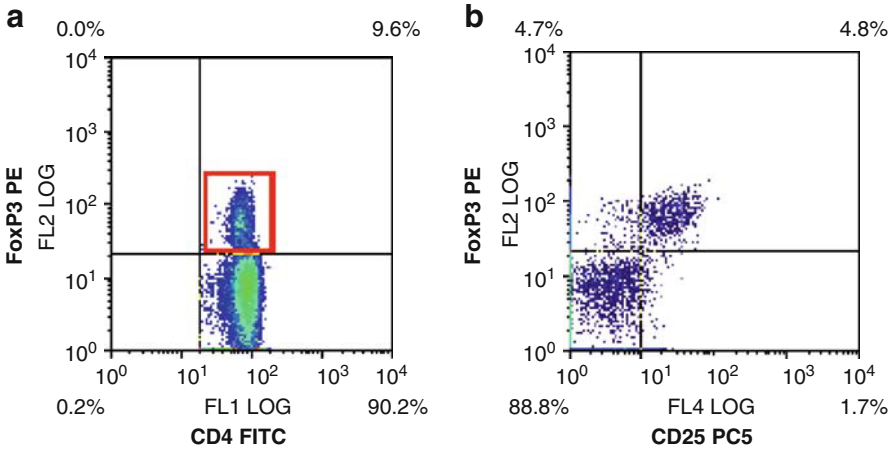

Fig. 1 Phenotypic analysis of regulatory T cells
a. The characteristic phenotype is of CD4+FoxP3+ cells. b. FoxP3 expression correlates with high expression of CD25.

2 The Origin of T-regulatory Cells: Thymically-derived or Generated in the Periphery?

Much has been made of the concept that most regulatory cells are derived by a discrete developmental pathway within the thymus which leads to a committed population of "natural" regulatory cells which never have the capacity to act as typical effector populations. Whilst much of this work has, necessarily, been performed in murine models, it has been much more difficult to determine that this is the case within human individuals. Interestingly, studies from both young and aged mice have shown that CD4+ FoxP3+ T-cells are found in both cohorts with some evidence of increased production during ageing. This raises the question as to whether CD4+ FoxP3+ T-cells continue to be produced by the human thymus throughout life. At first sight this might be considered unlikely given the well documented process of thymic involution that occurs during healthy human ageing, although it should be appreciated that the deterioration in naïve T-cell output is much more marked for the CD8+ population in comparison to CD4+ subsets. The reason for this last observation has never been entirely explained and could conceivably be partly explained by some contribution of ongoing CD4+ regulatory production.

However, it is also now clear that T-regulatory cells can be induced within the peripheral immune system by derivation from naïve or effector CD25- T-cell populations [9] (Fig. 2). The conditions that give rise to the generation of regulatory

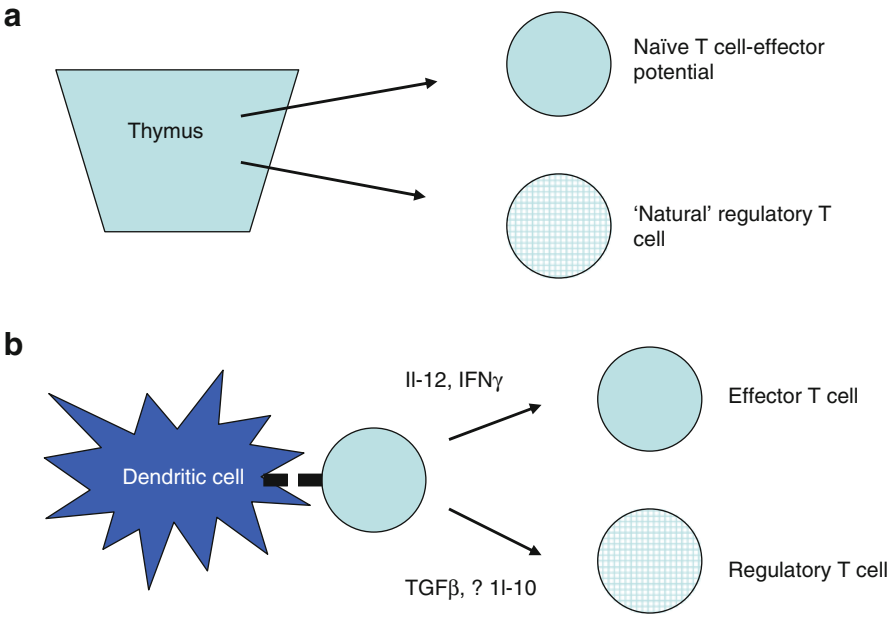

Fig. 2 Model for generation of T regulatory cells
Regulatory cells might arise from (a) a discrete pathway of differentiation through the thymus or (b) be induced from naïve T cell populations during T cell priming.

cells have not been entirely resolved, but are likely to involve microenvironmental signals including the presence of immunosuppressive molecules such as TGFβ and Il-10 at the time of dendritic cell priming of T-cell effector molecules [10]. When present during the first 3 days of T-cell priming the presence of TGFβ can induce FoxP3 expression in up to 90% of responding T-cells and this expression can be sustained for long periods of time in at least a subset of this population [11]. This mechanism of T-regulatory cell production is likely to become increasingly important during ageing of human populations. Given the longevity of humans and the well characterized involution of thymic function that occurs, it would appear highly unlikely that a discrete pathway of thymic maturation could provide the only source of T-regulatory cell production in adult life. Moreover, recent evidence has emerged to suggest that the turnover of T-regulatory cells is actually surprisingly short and that these cells are a highly proliferative population with short telomeres and great susceptibility to apoptosis [12]. Taken together, this evidence does strongly suggest that the peripheral production of T-regulatory cells within the immune system is a powerful factor in their generation. The site of origin of regulatory cell induction is also likely to be important in the functional properties of the mature cell as evidence is accumulating that extrathymic maturation is associated with impaired proliferative potential [13].

The number of CD4+ regulatory cells within the human immune system is variable depending both on the precise definition of such cells, the age of the individual and any underlying disease processes. Typically, between 2 and 5% CD4+ T-cells will express a CD25+ or FoxP3+ phenotype and further studies are required to show whether these numbers are influenced by any genetic or environmental factors.

3 T-regulatory Cell Populations During Healthy Ageing

An important question in relation to immune senescence is how the number and function of T-regulatory cells is influenced by physiological ageing, both in humans and in murine models. T-regulatory function certainly appears to be required throughout life and depletion of FoxP3+ T-cells in mice leads to a breakdown of self-tolerance at all ages [14]. Within murine systems, a number of investigators have shown an apparent increase in the number of CD4+ FoxP3+ T-cells in aged mice. CD4+FoxP3+ T-cell numbers can increase early in murine development with the BDC2.5NOD mouse showing an increase between the age of 6 and 18 weeks. However a 4 month old mouse is not aged in terms of the natural murine lifespan so this observation has limited relevance to studies of immune senescence. Zhao et al. studied Balb/c mice that were over 20 months of age and therefore represented a physiologically aged cohort [15]. In a comprehensive study they showed that the number of CD4+CD25+FoxP3+ T-cells increased with age in virtually all lymphoid compartments. However, the regulatory function of these cells did show some deficiencies in comparison to cells from young mice. In particular there was reduced inhibition of cytokine production by effector cells despite comparable sup-

pression of proliferation. It is interesting that the proportion of natural T-regulatory cells within the thymus can also increase during ageing suggesting that this mechanism may continue to make a contribution to peripheral accumulation [16]. Thymic-regulatory cells from elderly mice were able to suppress effector T-cells from young animals with similar efficiency to regulatory cells from younger mice. However, their ability to suppress effector cells from elderly mice was impaired indicating some functional differences between cells from young and older animals.

Sakaguchi's group has also addressed accumulation of T-regulatory cells during murine ageing and identified only a marginal increase in natural CD4+CD25+FoxP3+ T-cells. In contrast, increased levels of FoxP3 expression were seen in the CD4+CD25- population and identified a population with hyporesponsive function [17]. Their interpretation was that decreased immune function during ageing was primarily a reflection of a decrease in effector cell function rather than regulatory cell accumulation [18]. Comparable findings have been demonstrated in human subjects.

A number of studies are now addressing the number and function of T-regulatory cells in adult human populations. Our own data published in 2005 used CD4+CD25^{high}+CD45RO+ membrane phenotype as a marker of regulatory cell function [19]. Somewhat to our surprise at that time, we observed a significant increase in the regulatory T-cell population during ageing. In addition, we showed that the functional activity of these populations was also maintained during the ageing process with equivalent suppressor activity to that seen in young patients on a cell for cell basis. There are a number of methods by which T-regulatory function may be assessed, but suppression of interferon-γ cytokine production by effector T-cells in coculture experiments is one of the most reliable and sensitive methods. Suppression of proliferation of effector cells in antigenic stimulation or allogenic mixed lymphocyte cultures can also be a valuable test.

Other investigators have also addressed the question of T-regulatory cell numbers in human ageing [20, 21] and have demonstrated that CD4+ FoxP3+ T-cells are seen to increase during ageing [22]. This is reassuring as it indicates that the more contemporary phenotype of regulatory cells, namely FoxP3 expression, can also be used to corroborate the increase in regulatory cells with ageing. Despite this, some evidence continues to suggest that functional activity is not equivalent in older subjects [23].

An important question that needs to be addressed in T-regulatory cell biology is the degree of coexpression of FoxP3 and CD25 on CD4 regulatory populations. In particular, CD25- T-cells expressing FoxP3 are observed in a number of clinical settings and may be related to the use of immune-suppression, ongoing chronic activation, or potentially immune senescence. There have been few studies to address this within humans although within murine systems an increase in CD25- FoxP3+ regulatory cells with ageing has been observed. In general, the evidence seems to suggest that FoxP3 is the more reliable marker of functional regulatory activity and the expression of CD25 therefore becomes less significant. CD25 is the high affinity receptor for interleukin 2 and its physiological role in T-regulatory functions has never been completely explained. Some authors have suggested that CD4+ CD25+

T-cells act as a "sink" for cytokine production and thereby act to limit cellular proliferation within the microenvironment but this proposal has not yet received substantial and confirmatory support.

The accumulation of regulatory cells with ageing does suggest that there may be increased generation of regulatory cells within the peripheral immune system in association with ageing. The mechanisms that may underlie this are unknown but differences have been reported between signalling processes in dendritic cells from young and old donors [24]. Reduced phosphorylation of AKT within DC in response to cell stimulation may be one reflection of differential signalling responses in these cells from elderly donors.

As with effector populations, T-regulatory cells can be divided into naïve and memory subsets based on their expression of CD45 isoforms. Naive regulatory populations characterized by CD45RA expression are predominant in infants but are still detectable in adult subjects [25]. The extent to which these remain in elderly donors with immune senescence has yet to be studied.

4 Regulatory T-cell Populations within the CD8+ T-cell Repertoire

In contrast to the extensive investigation of CD4+ regulatory T-cells there has been much less study of regulatory populations on cells within the CD8+ population. Suppressor T-cells have been described for many years and their phenotype appears to be dominated by lack of expression of the CD28 costimulatory molecule whereas expression of FoxP3 on CD8 has been poorly characterized. Naive CD8+ T-cell numbers decline dramatically with age to be replaced by accumulation of effector populations and this homeostatic instability may contribute to the differential structure of CD4 and CD8 T-cell pools [26].

A complexity is that FoxP3 may be expressed transiently after T-cell activation of effector T-cells although this induction does not lead to acquisition of complete regulatory function [27]. CD8+ populations that do express FoxP3 have been reported in a number of clinical scenarios [28, 29] but their functional role, and relevance to ageing, has not yet been addressed.

5 The Potential Contribution of T-regulatory Cells to Immune Senescence

One area in which there has been disappointing progress in T-regulatory biology has been confirmation of the antigenic specificity of these populations. Great debate continues to address the issue as to whether effector CD4+ T-cells and CD4+ regulatory cells recognize the same population of antigenic determinants or display specificity against discrete epitopes which may themselves play a role in determining

the physiological direction of T-cell differentiation [30]. T-regulatory cells do appear to have specificity for self-epitopes and in this regard overlap at least partially with a population of potentially self reactive effector cells [31]. The former hypothesis gains considerable import from use of T-cell receptor transgenic models in which alteration of environmental conditions can lead to the generation of T-regulatory or T-effector cells with the same T-cell receptor expression. It had been believed that T-regulatory cells may represent a population with uniquely high affinity for self-antigen but such experiments suggest that critical determinants during T-cell priming maybe more important in determining the nature of T-cell differentiation after antigen priming.

Despite this, it has been widely reported that, although T-regulatory cells may require antigenic stimulation for activity, their activity, once triggered, is non-specific in nature and therefore can act to suppress bystander populations. In this regard, it is entirely possible that individuals who accumulate large populations of T-regulatory cells can be subject to some form of generalized immune suppression which may impact on their ability to mount effective immune responses against infectious agents or, potentially, tumour cells. There is great interest in studying the number and function of T-regulatory cells in patients with active malignant disease and a number of trials and animal models, either targeting CD25 or CTLA4, are now addressing how suppressional manipulation of T-regulatory cell function may serve to increase antigen-specific T-cell immune responses against transformed tissue [32, 33]. Some of these studies are already providing encouraging clinical responses but, perhaps not surprisingly, such treatments can often unleash powerful autoimmune phenomena with unusual antigenic specificities, including hypophysitis or hepatitis [34].

The question therefore needs to be addressed as to whether the increase in T-regulatory cells that is observed during healthy ageing contributes directly to immune senescence in this population [35]. In contrast, despite an increase in absolute numbers the suggestion that regulatory cell function may be impaired in ageing might also be relevant to the increase in autoimmune disease with age. To date there has been little experimentation which has allowed any detailed interpretation of this concept. However, Trzonkowski et al. have shown that the accumulation of regulatory cells that is observed with ageing is directly associated with impaired activity of CD8+ and NK-cells [21]. When effector cells were purified away from regulatory populations they regained their natural level of activity whereas add-back of regulatory populations led to reestablishment of impaired function. Within the murine system it should be possible to deplete these populations and assess T-cell immunity in mice of different ages and these experiments are eagerly awaited. Sharma et al. have shown that the accumulation of regulatory T-cells in aged Balb/c mice is directly responsible for the inability of older animals to reject tumour tissue [36]. Encouragingly, depletion of regulatory T-cells with an anti-CD25 antibody was able to reverse this deficiency.

Within the human system, such experiments are clearly much more difficult to devise but there is surely enough evidence now to make this an important clinical aim. Useful information is likely to be gleaned from the increasing number of early

phase trials of T-regulatory modulation in both malignant and infectious disease which will reveal something regarding contribution of T-regulatory cells to immune senescence. Therapeutic manipulation of T-reg numbers will prove a difficult challenge but the use of agents such as interleukin-7 may modulate the nature of thymic output [37]. Granulocyte colony stimulating factor (G-CSF) administration leads to increased recruitment of regulatory cells through activation of tolerogenic plasmacytoid dendritic cells [38] and this cytokine axis might therefore offer scope for intervention. Adrenergic innervation of the thymus has been postulated to play a role in thymic involution and pharmacological blockade leads to increased T-regulatory cell production [39]. One interesting question will be the relative serum level and tissue availability of TGFβ during ageing as this may play an important role in the peripheral accumulation of regulatory cells.

6 Conclusion

In conclusion, T-regulatory cells are emerging as the most intensively studied population of cells within the human immune system at the current time. As such, it is not surprising that they are now being addressed in relation to immune senescence, itself one of the most important areas of clinical immunology. Initial data suggests that regulatory cell numbers are indeed increased in healthy elderly individuals, and that the functional properties of these cells are also maintained. Although more needs to be discovered regarding the antigenic specificity and functional activity of these cells, it is likely that manipulation of T-regulatory cell function could represent a novel form of immunotherapeutic intervention in elderly individuals. Whether or not it will be possible to limit regulatory cell function without an associated increase in autoimmunity is currently unknown. Such clinical intervention is likely only to be possible when much greater understanding is made of the natural physiological role of these cells in immune responses.

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Age-related Changes in Subpopulations of Peripheral Blood Lymphocytes in Healthy Japanese Population

Masanori Utsuyama, Yuko Kikuchi, Masanobu Kitagawa and Katsuiku Hirokawa

Contents

Abstract: Peripheral blood mononuclear cells were obtained from healthy Japanese individuals ranging in age from 20 to 90 years old and analyzed by using three color flow cytometer with regards to the number and percentage of various lymphocytes. In addition, we assessed the proliferative capacity of T-cells in the presence of an anti-CD3 monoclonal antibody and the amount of cytokines produced in the supernatant.

The results showed that an age-related decline was observed in the numbers of $CD3^+$ T-cells, $CD8^+$ T-cells, naive T-cells, $CD8^+CD28^+$ T-cells, and B-cells and in the proliferative capacity of T-cells. The rate of decline in these immunological parameters except for the number of $CD8^+$ T-cells was steeper in males than in females ($p<0.05$). An age-related increase was observed in the number of $CD4^+$ T-cells, memory T-cells, and NK-cells and in the CD4/CD8 ratio The rate of increase of these immunological parameters was steeper in females than in males ($p<0.05$). The T-cell proliferation index (TCPI), which was calculated based on T-cell proliferative activity and the number of T-cells, showed an age-related decline. The rate of decline in the TCPI was again steeper in males than in females ($p<0.05$). The score of immunological vigor calculated using 5 T-cells parameters also declined with age, and the rate of decline was steeper in males than in females ($p<0.05$). The

K. Hirokawa (✉)
Institute for Health and Life Sciences
Ascent Myogadani 4F
4-6-22, Kohinata, Bunkyo-ku
Tokyo 112-0006, Japan
Tel: 81-3-6820-6139
E-mail: hirokawa@h-ls.jp

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present study has confirmed the age-related changes in immunological parameters reported in literature. In addition, we found that a statistically significant difference was observed between males and females in some immunological parameters such as the number of T-cells and TCPI. The slower rate of decline in the immunological parameters studied in females than in males may be consistent with the fact that women survive for longer period of time than men.

1 Introduction

Immunological functions are known to decline with age in many animal models and humans (Linton and Dorshkind 2004; Utsuyama et al. 1992; Hirokawa et al. 2006). Understanding the level of immunological functions at an individual level is clinically important, since the immunological decline is accompanied by various diseases such as infections, cancer and vascular diseases.

Accumulating evidences mainly obtained from animal models have shown that age-related immunological decline mainly occurs in T-cell dependent immune functions, and is mainly caused by thymic involution that begins in the early phase of life (Hirokawa et al. 2006).

In humans, data regarding immunological functions are mainly obtained from blood serum and blood cells. Serum contains immunoglobulins, complements and cytokines. The levels of IgG and IgA in serum show a trend of increase with age (Suzuki et al. 1984). The level of complements does not change remarkably with age. The level of cytokines in healthy people is generally low. In contrast, the level of white blood cells (WBC) changes remarkably during disease and also with aging. WBC comprises granulocytes, lymphocytes and monocytes. There are various subpopulations of lymphocytes with different functions. Data regarding the age-related changes in lymphocytes and their functions are not sufficiently available as yet.

The purpose of this study is to provide immunological data on peripheral blood lymphocytes obtained from 162 male and 194 female healthy volunteers, ranging in age from 20 to 90 years. Our study discusses the age-related changes in subpopulations of peripheral blood lymphocytes from both immunological and gerontological viewpoints.

2 Materials and Methods

Blood specimens: Two milliliters of blood was taken in a tube containing ethylenediaminetetraacetic acid (EDTA-2K) for hematological analysis performed using a PENTRA80 analyzer (Horiba, Kyoto, Japan). Eight milliliters of blood was taken in a cell preparation tube (vacutainer, 362761, Becton Dickinson (BD), NJ) for collecting mononuclear cells and was used for immunological analyses.

Subjects: Healthy volunteers were selected based on clinical records and laboratory examinations. None of the blood donors were suffering from neoplastic or autoimmune disease; further, none were receiving any medications that could

Table 1 Number of male and female subjects

Age	20 – 29	30 – 39	40 – 49	50 – 59	60 – 69	70 – 79	80 –	Total
Male	13	23	35	37	29	22	3	162
Female	44	32	36	34	18	26	4	194

influence immune functions. Routine laboratory examinations of the serum were performed to examine the liver and kidney functions. A total of 162 males and 194 females were examined in the present study. Table 1 shows the number of male and female subjects and their ages.

Flow cytometry: Mononuclear cells that were obtained from the peripheral blood, as described above, were stained with a combination of 2 or 3 monoclonal antibodies (mAbs) conjugated with 2 or 3 chromophores. A fluorescence-activated cell sorting flow cytometer (FACScan BD) was employed in the present study.

Monoclonal antibodies: The antibodies used were fluorescein isothiocyanate (FITC) conjugated anti-CD4, FITC-conjugated anti-CD20 and FITC-conjugated anti-CD16; phycoerythrin (RD1) conjugated anti-CD3, RD1-conjugated anti-CD8 and RD1-conjugated anti-CD25; phycoerythrin-Texas Red (ECD) conjugated anti-CD45RA and ECD-conjugated anti-CD3; phycoerythrin-cyanin 5.1 (PC5) conjugated anti-CD28: phycoerythrin (PE) conjugated anti-CD56. Those mAbs were purchased from Beckman Coulter. The following combinations of mAbs were used: CD3-RD1/CD20-FITC, CD4-FITC/CD8-RD1/CD45RA-ECD, CD4-FITC/CD8-RD1/CD28-PC5, CD56-PE/CD16-FITC, CD3-ECD/CD4-FITC/CD25-RD1.

Proliferative response of T-cells: The proliferative response of T-cells to anti-CD3 mAb (ORTHOCLONE OKT3, ORTHO BIOTEC, NJ) was assessed according to MTS method (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Co., WI)).

Assays were performed in microplates (3860-096, Asahi Glass Co. Japan). The cells (1×10^5) in 0.2 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) were stimulated with immobilized anti-CD3 mAb (Orthoclone OKT3, Ortho Biotec, NJ). The plates were then placed in a 5% CO_2 incubator for 72 hrs. After incubation for 68 hrs, 40 µl of MTS solution (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Co., WI)) was added into each well and absorbance at 490nm was recorded with a spectrophotometric plate reader; this value was used for determining the relative magnitude of T-cell proliferation.

T-cells proliferation index (TCPI) and immunological age (IA): TCPI was calculated by the following equation.

TCPI = T-cell proliferative activity × (T-cell number/1000)

In this equation, T-cell proliferative activity was obtained as optical density (OD_{490}) ranging between 0.95 and 2.0 by the abovementioned MTS method. The TCPI and age showed a statistically significant correlation: TCPI = −0.0174 x (Age)+ 2.5348 (Fig. 5c and 5d). Using this equation, it is possible to calculate age by assigning a value to TCPI. The age calculated by this equation was referred to as immunological age (IA).

Scoring and grading of immunological functions: The values of immune parameters were standardized by assigning scores of 3 (high level), 2 (moderate level) and 1

Table 2 Scoring and grading of immunological vigor

Scoring		
SIV-7 7 parameters	SIV-5 5 parameters	Grading
21	15	Grade V Sufficiently high
20 ~ 18	14 ~ 13	Grade IV Safety zone
17 ~ 14	12 ~ 10	Grade III Observation zone
13 ~ 10	9 ~ 7	Grade II Warning zone
9 ~ 7	6 ~ 5	Grade I Critical zone

(low level) according to the data base obtained from 300 healthy people. After standardization, the scores of different types of immune parameters were summed and the numerical value obtained for each individual was termed the score of immunological vigor (SIV). These scores were then classified into 5 grades, as shown in Table 2.

SIV-7 comprises 7 parameters that are number of T cells, TCPI, CD4/CD8 ratio, number of naïve T cells, naïve/memory ratio, number of B cells and number of NK cells. SIV-5 comprise 5 T cell-related parameters that T cells, TCPI, CD4/CD8 ratio, number of naïve T cells and naïve/memory T cells ratio.

Assessment of cytokine production: Assays were performed in microplates (3860-024, Asahi Glass Co. Japan). Cells (1×10^6) in 1.5 ml of RPMI 1640 supplemented with 10% FBS were stimulated with immobilized anti-CD3 mAb (Orthoclone OKT3, Ortho Biotec, NJ). Culture supernatant were collected at 48 hrs and stored at -80°C until use. A flow cytomix kit (BMS810FF, Bender MedSystems, Austria) was employed for the evaluation of cytokines (Interleukin (IL)-1β, IL2, IL-4, IL5, IL-6, IL-8, IL-10, IL-12/p70, interferon (IFN) γ, tumor necrosis factor (TNF) α, TNFβ and the assessment was performed using a FACScan analyzer.

Statistical Analysis: All statistical analyses were performed using StatView software. Statistical significance was defined as $p < 0.05$. Gender difference was examined by SMA analysis.

3 Results

3.1 Number of Whole WBCs, Red Blood Cells and Lymphocytes in the Blood

The number of red blood cells (RBC) showed a significant age-related decrease ($p < 0.001$) in males and a declining trend with age in females (p=0.9535) (Fig. 1a and 1b) (Table 3). The difference between males and females with regard to the age-related decline in the number of RBC was statistically significant ($p < 0.001$). Although an age-related decline was observed in males, but not in females, the absolute level of RBC was higher in males than in females regardless of age.

The number of WBCs including granulocytes, lymphocytes and monocytes showed a declining trend with age in both males (p=0.0824) and females (p=0.2588); no statistically significant difference was observed between males and females in this regard (Fig. 1c and 1d) (Table 3).

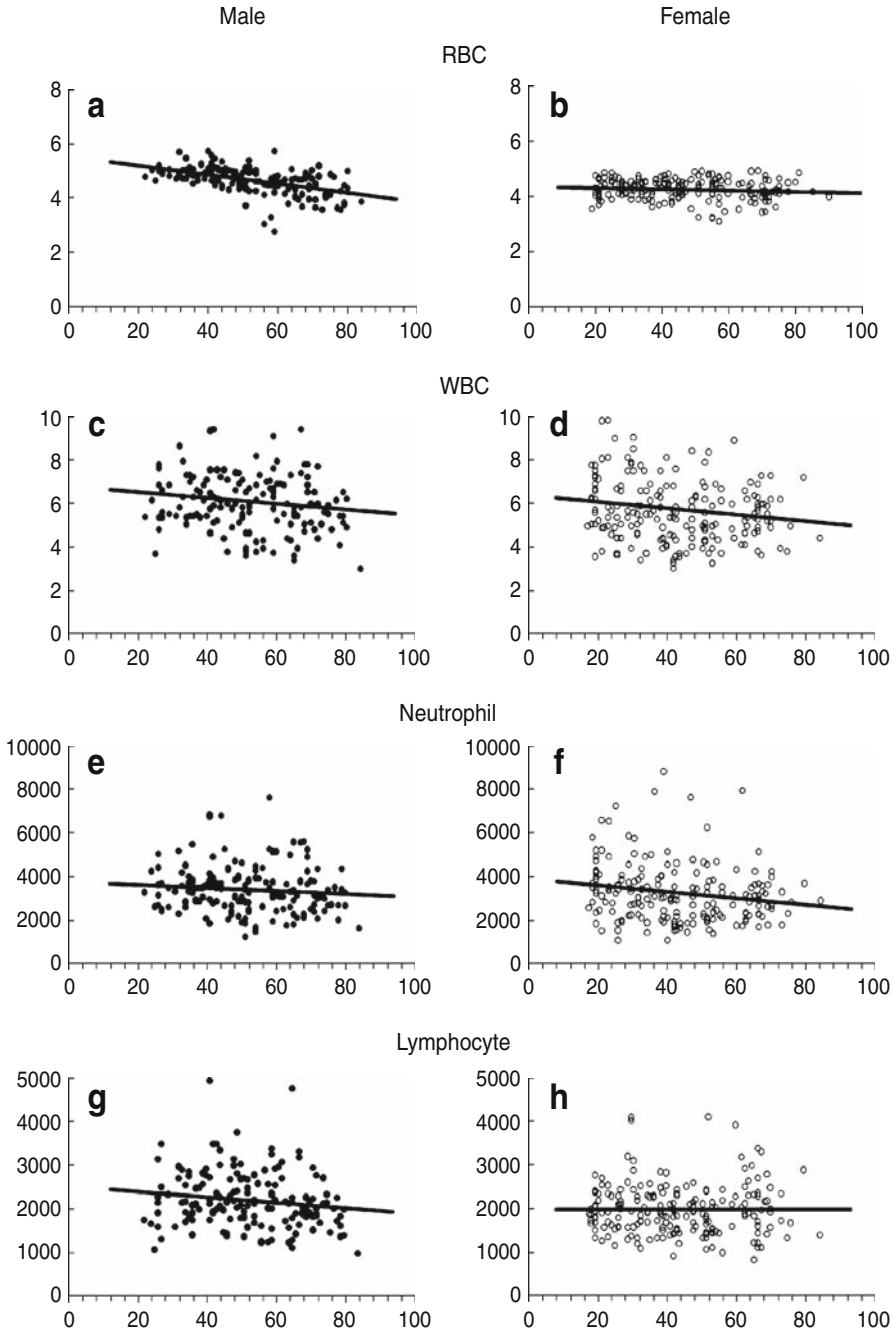

Fig. 1 Age related changes in the number of red blood cells (RBC) in males (a) and females (b), white blood cells (WBCs) in males (c) and females (d), neutrophils in males (e) and females (f), and lymphocytes in males (g) and females (h)

Table 3 Results of regression analysis on age and immunological parameters

	Male (N=162)			Female (N=194)			SMA[§]
	Regression curve	R*	p value	Regression curve	R*	p value	analysis
RBC	-0.017x + 5.544	0.517	<0.0001	-0.0002x + 4.264	0.004	0.9535	0.001
Lymphocytes[#]	-6.430x + 2526	0.149	0.0593	+1.038x + 1934	0.031	0.6637	0.015
T-cells[#]	-6.150x + 1791	0.186	0.0176	-2.390x + 1508	0.111	0.1249	0.049
$CD4^+$ T-cells[#]	+0.024x + 903.3	0.001	0.9897	+1.962x + 769.6	0.116	0.1075	0.005
$CD8^+$ T-cells[#]	-4.564x + 719.3	0.286	0.0002	-4.048x + 649.6	0.306	<0.0001	NS
CD4/CD8 ratio	+0.027x + 0.839	0.316	<0.0001	+0.029x + 0.898	0.452	<0.0001	0.003
Naïve T-cells[#]	-1.365x + 471.3	0.089	0.2615	-0.598x + 439.5	0.055	0.4470	0.004
Memory T-cells[#]	+1.392x + 431.8	0.113	0.1531	+2.557x + 330.1	0.253	<0.0001	NS
Naïve / Memory ratio	-0.005x + 1.081	0.159	<0.0001	-0.007x + 1.356	0.258	0.0003	NS
MTS (OD_{490})	-0.006x + 1.629	0.314	<0.0001	-0.004x + 1.544	0.224	0.0017	NS
Proliferation Index	-0.016x + 2.817	0.289	0.0002	-0.008x + 2.306	0.190	0.0080	0.010
SIV[##] (5 items)	-0.036x + 14.01	0.262	0.0008	-0.035x + 14.07	0.334	<0.0001	0.012
B-cells[#]	-1.844x + 266.0	0.167	0.0336	-0.444x + 148.8	0.103	0.1525	0.001
NK-cells[#]	+1.787x + 368.6	0.103	0.1935	+3.208x + 218.5	0.269	0.0002	0.001
[$]$CD8^+CD28^+$ cells[#]	-6.089x + 597.0	0.543	<0.0001	-4.136x + 476.9	0.477	<0.0001	0.038
WBC[#]	-0.014x + 6.801	0.137	0.0824	-0.007x + 6.118	0.082	0.2558	NS
Neutrophils[#]	-6.942x + 3719	0.097	0.2178	-8.535x + 3729	0.116	0.1063	NS
SIV[##] (7 items)	-0.039x + 19.65	0.270	0.0005	-0.032x + 19.17	0.264	0.0002	NS
[$]Regulatory T-cells[#]	+0.042x + 46.01	0.026	0.7502	+0.132x + 36.32	0.100	0.1818	NS

#: Number / mm^3 . *: R, Correlation coefficient. §: SMA Standardized Major Axis Test,

##: SIV: Scoring of immunological vigor

$: Number of $CD8^+$ $CD28^+$ cells was obtained from 107 males and 103 females.

$$: Number of regulatory T-cells ($CD4^+$ $CD25^+$) was obtained from 154 males and 181 females.

The number of neutrophils showed a decreasing trend with age in both males (p=0.2178) and females (p=0.1063); no significant difference was observed between males and females (Fig. 1e and 1f) (Table 3).

The number of lymphocytes showed a decreasing trend with age in males (p=0.0593) and an increasing trend with age in females (p=0.1249); statistically significant difference was observed in the age-related change between males and females (p=0.015) (Fig. 1g and 1h) (Table 3).

3.2 Flow Cytometric Analysis

(a) $CD3^+$ T-cells.

The number of $CD3^+$ T-cells showed a statistically significant decrease with age in males (p =0.0186), and a decreasing trend with age in females (p =0.1249). The difference in the age-related change in the number of $CD3^+$T-cells between males and females was statistically significant (p=0.049) (Fig. 2a and 2b) (Table 3).

(b) $CD4^+$ T-cells.

The number of $CD4^+$ T-cells showed an increasing trend with age in both males (p=0.9897) and females (p=0.1075). This trend was greater in females than in males, and the difference between males and females with regard to this trend was statistically significant (p=0.005) (Fig. 2c and 2d) (Table 3).

(c) $CD8^+$T-cells.

The number of $CD8^+$T-cells showed an age-related decrease in both males ($p < 0.0002$) and females ($p < 0.0001$), but no difference was observed between males and females with regard to this decrease (Fig. 2e and 2f) (Table 3).

(d) The ratio of $CD4^+$ T-cells to $CD8^+$ T-cells (CD4/CD8 ratio).

The CD4/CD8 ratio increased with age in both males (p <0.0001) and females ($p < 0.0001$), and this increase was significantly greater in females than in males ($p < 0.003$) (Fig.2g and 2h) (Table 3).

(e) $CD8^+CD28^+$ T-cells.

The number of $CD8^+CD28^+$ T-cells showed an age-related decrease in both males (p <0.0001) and females (p <0.0001) (Fig. 3a and 3b), and the rate of this decline was more pronounced in males (-6.089) and in females (-4.136) ($p < 0.003$) (Table 3).

(f) $CD4^+CD45RA^+$ naïve T-cells.

The number of $CD4^+CD45RA^+$ naïve T-cells showed a decreasing trend with age in both males (p=0.2615) and females (p=0.4470) (Fig.3c and 3d). This decreasing trend was greater in males than in females, and the difference between males and females was statistically significant (p=0.004).

(g) $CD4^+CD45RO^+$ memory T-cells.

The number of $CD4^+CD45RO^+$ memory T-cells showed an increasing trend with age in males (p=0.1531), and an age-related increase in females (p=0.0001) ($p < 0.0001$) (Fig. 3e and 3f). In this case, there is no significant gender difference (Table 3).

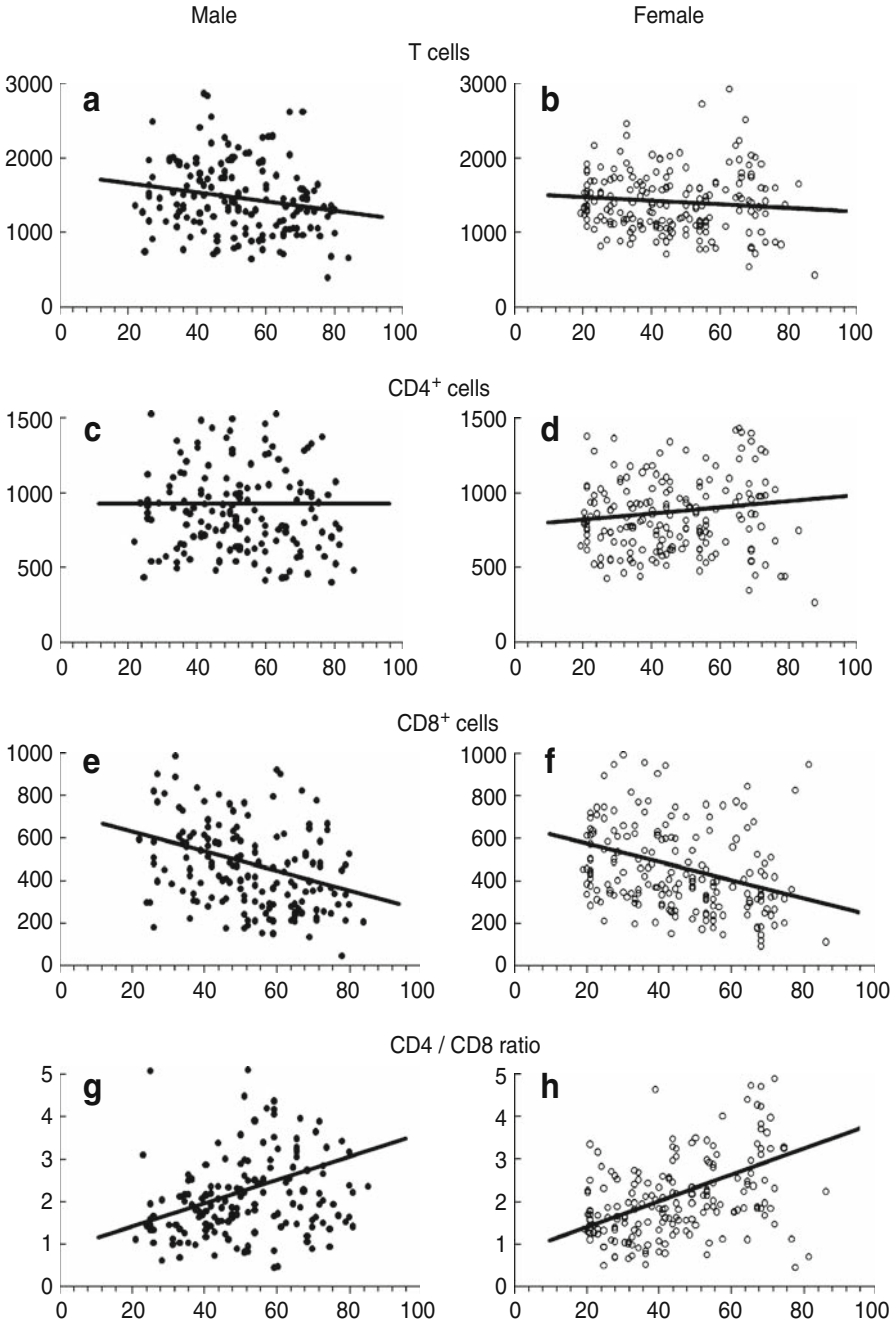

Fig. 2 Age related changes in the number of T-cells in males (a) and females (b), $CD4^+$ T-cells in males (c) and females (d), $CD8^+$ T-cells in males (e) and females (f), and the CD4/CD8 ratio in males (g) and females (h)

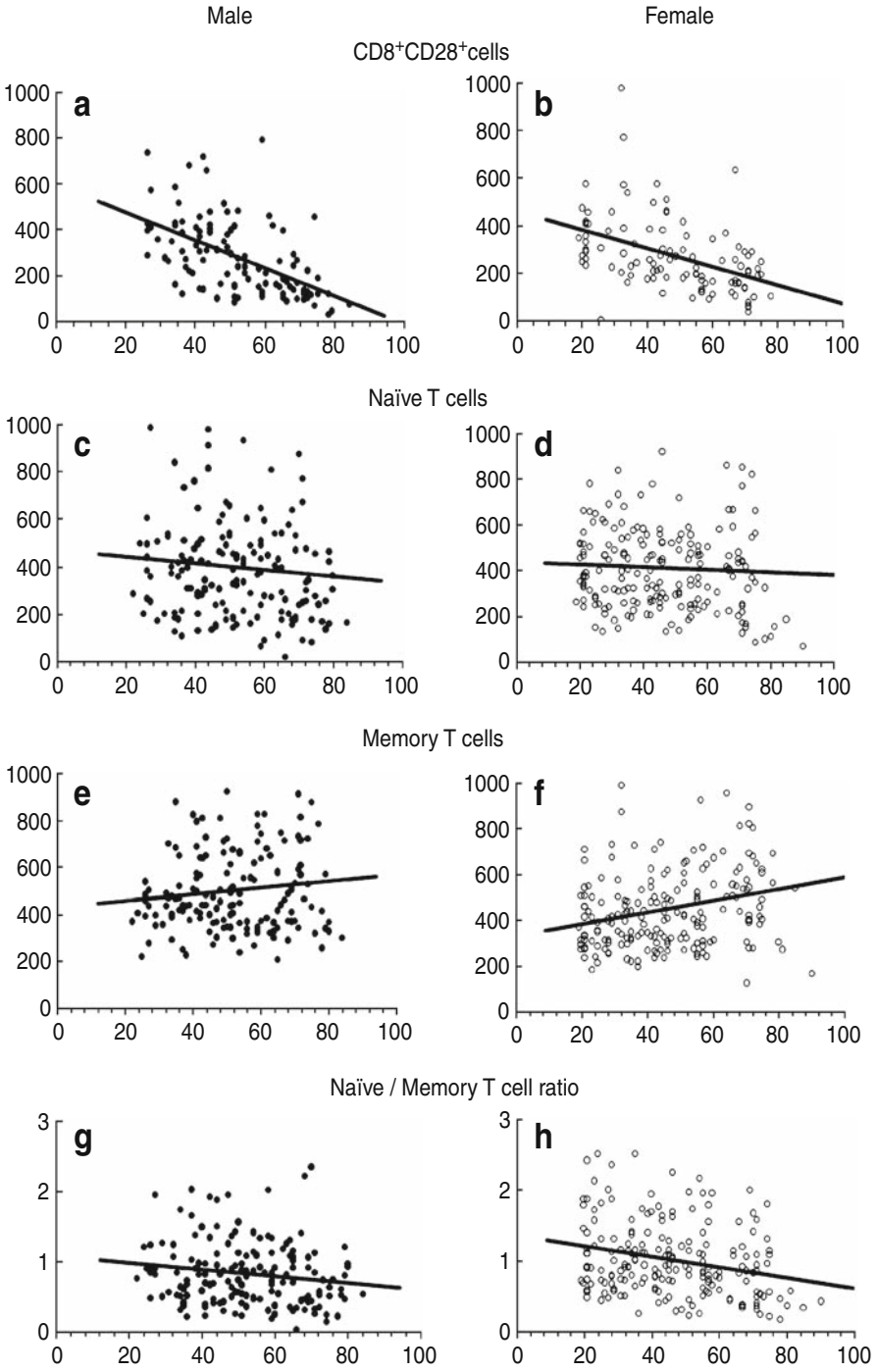

Fig. 3 Age related changes in the number of $CD8^+CD28^+$ T-cells in males (a) and females (b), naive T-cells in males (c) and females (d), memory T-cells in males (e) and females (f), and native to memory T-cells (N/M) ratio in males (g) and females (h)

(h) Ratio of naïve to memory T-cells.

The naïve to memory T-cells (N/M) ratio showed an age-related decrease in both males (p <0.0001) and females (p <0.0003), and this decrease was statistically significant (Fig. 3g and 3h). However, no significant gender difference was observed (Table 3).

(i) $CD4^+CD25^+$ T-cells.

The number of $CD4^+CD25^+$ T-cells showed an increasing trend with age in both males (0.7502) and females (0.1818) (Fig. 4a and 4b), but this increase was statistically not significant. Further, no gender difference was observed (Table 3).

(j) $CD20^+$ B-cells.

The number of $CD20^+$ B-cells showed a decrease with age in males ($p < 0.05$) and showed a decreasing trend with age in females (p =0.15) (Fig. 4c and 4d); no statistically significant difference was observed between males and females with regard to this decrease ($p < 0.001$) (Table 3).

(k) $CD56^+CD16^+$ NK-cells.

The number of $CD56^+CD16^+$ NK-cells showed an age-related increase in females (p <0.0002) and an increasing trend with age in males (p=0.19) (Fig. 4e and 4f). A statistically significant gender difference was observed (p <0.001) (Table 3).

3.3 Proliferative Response of T-cells

(a) Proliferative response of T-cells by anti-CD3 monoclonal antibody ($MTS\text{-}OD_{490}$).

The proliferative response of T-cells was measured by MTS method and was expressed as OD_{490}. It showed an age-related decrease in both males (p<0.0001) and females (p<0.002) (Fig. 5a and 5b), but no gender difference was observed.

(b) T-cell proliferation index (TCPI).

The TCPI showed an age-related decrease in both males ($p < 0.0002$) and females (p <0.008) (Fig. 5c and 5d). The decrease was more pronounced in males than in females ($p < 0.01$) (Table 3).

(c) Correlation between $CD8^+CD28^+$ T-cells and T-cell proliferative response ($MTS\text{-}OD_{490}$).

The number of $CD8^+CD28^+$ T-cells and $MTS\text{-}OD_{490}$ showed an age-related decease in both males and females; this decrease was statistically significant. It is interesting to note that a good correlation was observed between the number of $CD8^+CD28^+$ T-cells and $MTS\text{-}OD_{490}$ (Fig. 4g and 4h).

3.4 Scoring of Immunological Vigor (SIV)

(a) SIV-7.

SIV-7 was calculated based on 7 parameters: T-cells number, TCPI, CD4/CD8 ratio, naïve T-cell number, naive/memory T-cells ratio, B-cell number and NK-cell number.

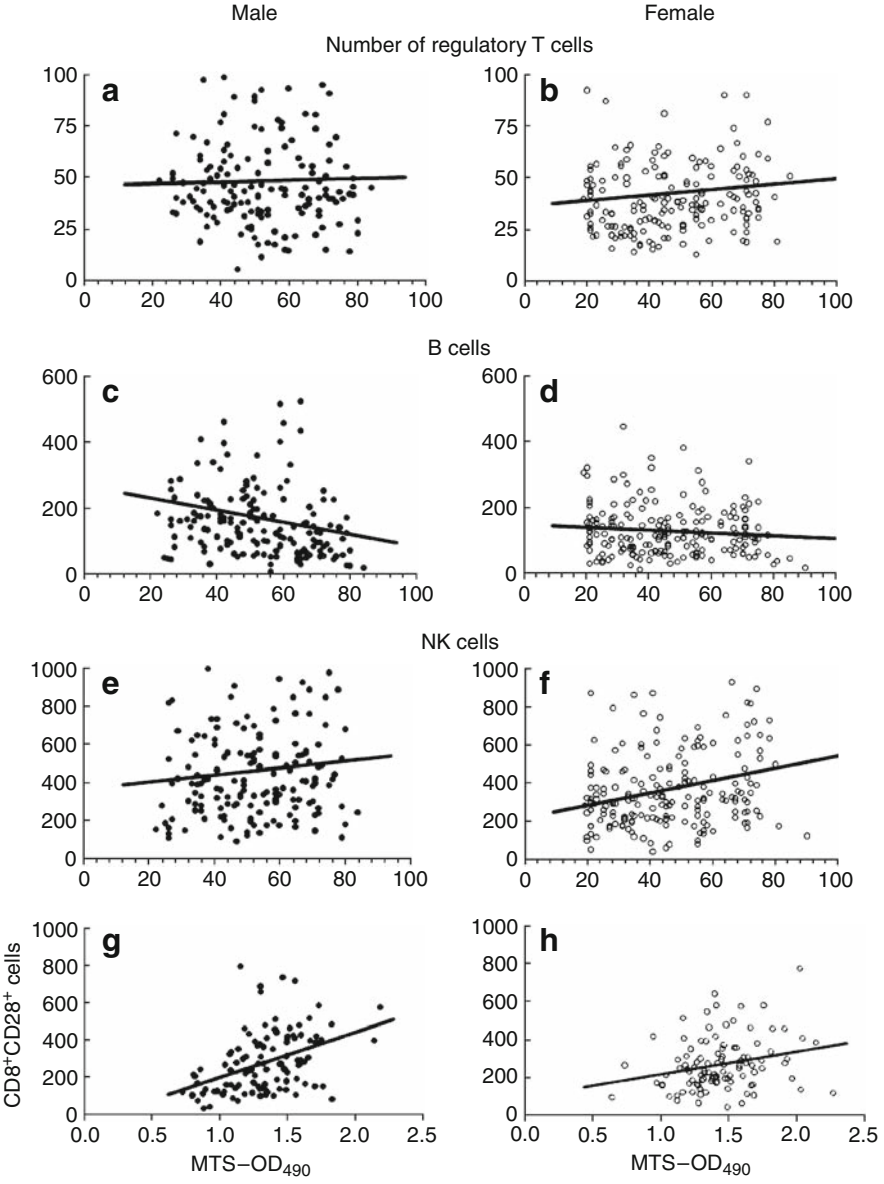

Fig. 4 Age related changes in the number of regulatory T-cells in males (a) and females (b), B-cells (WBC) in males (c) and females (d), NK-cells in males (e) and females (f), and the correlation between the number of $CD8^+CD28^+$ T-cells and the T-cell proliferative response (MTS-OD490) in males (g) and females (h)

SIV-7 showed an age-related decrease in both males ($p < 0.0005$) and females ($p < 0.0002$) (Fig. 5e and 5f). No gender difference was observed (Table 3).

(b) SIV-5.

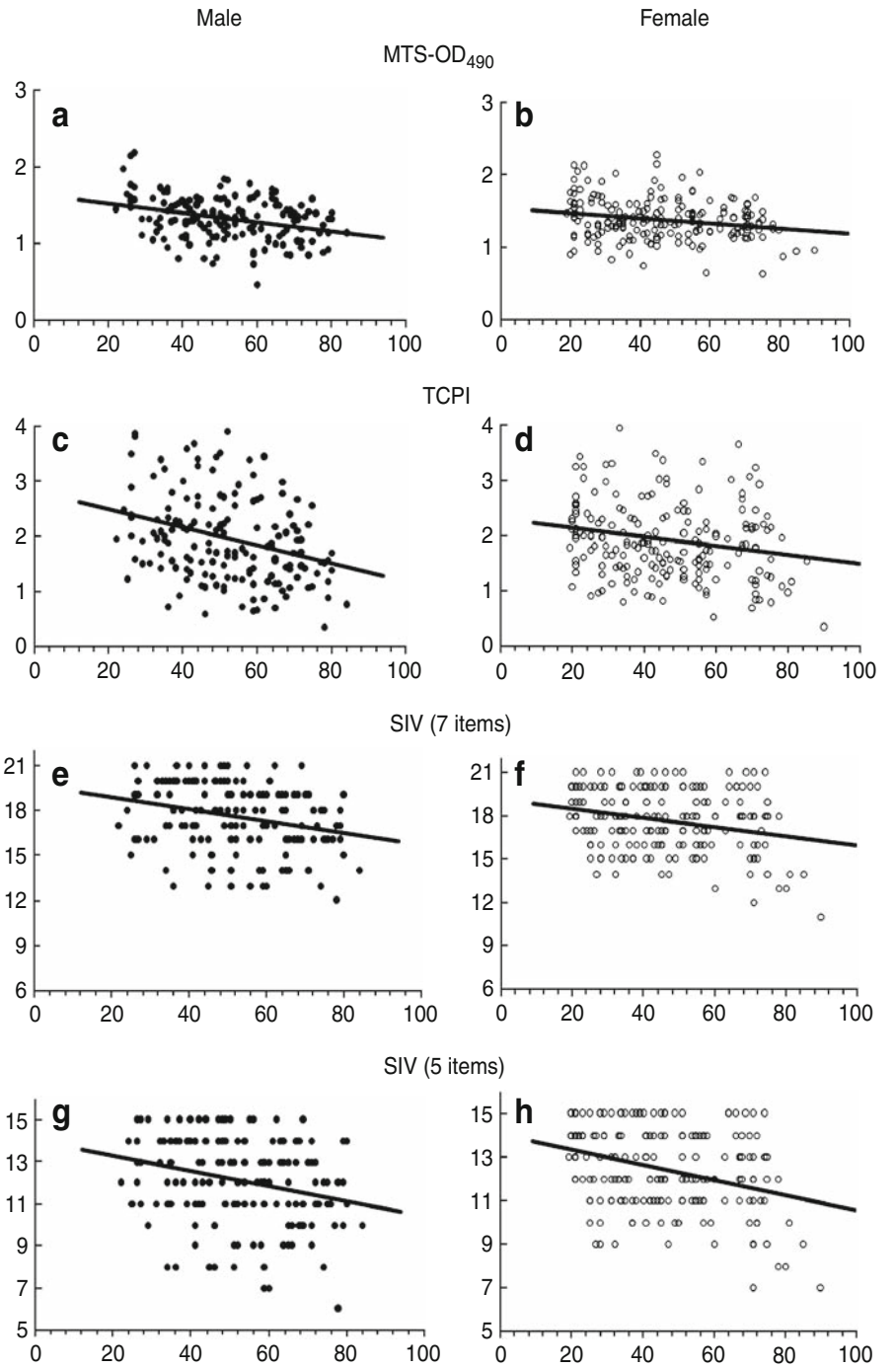

Fig. 5 Age-related change in the T-cells proliferative response (MTS-OD490) in males (a) and females (b), T-cell proliferation index (TCPI) in males (c) and females (d), score of immunological vigor (SIV)-7 in males (e) and females (f), and SIV-5 (T-cell immune score) in males (g) and females (h)

Table 4 Regression analysis on cytokine productions and age in males and females

	Males (N=64)			Females (N=49)			SAM[#]
	Regression curve	R*	p value	Regression curve	R*	p value	analysis
IFNγ	-10.29x + 0.227	2951	0.0707	-5.95x + 2823	0.123	0.3969	NS
IL-1β	-20.72x + 0.276	2553	0.0272	-17.27x + 2857	0.170	0.2419	NS
IL-2	-2.498x + 0.129	307	0.3477	-4.188x + 360	0.243	0.1077	NS
IL-4	-0.021x + 0.020	18.8	0.8853	+0.063x + 11.1	0.083	0.6010	NS
IL-5	+0.694x + 0.099	50.9	0.4468	+0.068x + 53.0	0.022	0.8880	NS
IL-6	-72.72x + 0.248	7713	0.0482	-36.90x + 9470	0.076	0.6054	p=0.010
IL-8	+ 4.94x + 0.133	2291	0.2939	+ 5.34x + 2073	0.150	0.3025	NS
IL-10	- 9.05x + 0.175	1282	0.1655	- 1.78x + 742	0.063	0.6656	p=0.004
TNFα	-144.5x + 0.192	24500	0.1411	-185.5x + 28125	0.251	0.1003	NS
TNFβ	-10.02x + 0.378	974	0.0017	- 3.48x + 596	0.222	0.1284	p=0.012

*R: Correlation coefficient. [#] SMA: Standardized Major Axis Test. NS: Not significant.

SIV-5 was calculated by using 5 parameters: T-cells number, TCPI, CD4/CD8 ratio, naïve T-cell number, and naive/memory T-cells ratio; SIV-5 is sometimes termed as T-cell immune score.

The T-cell immune score showed an age-related decrease in both males ($p < 0.001$) and females ($p < 0.0001$) (Fig. 5g and 5h). A more pronounced decrease was observed in males than in female ($p < 0.02$) (Table 3).

3.5 Cytokine Production

In the present study, lymphocytes were cultured in vitro in the presence of immobilized anti-CD3 mAb and the cytokines produced in the supernatant were assessed as described previously. The subjects for cytokine production comprised 64 males and 49 females; this sample size was not adequate for statistical analysis. This preliminary examination has revealed that an age related decrease in the levels of IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, TNFα and TNFβ in both male and female subjects. In contrast, an age-related increase was observed in IL-8 (Table 4).

3.6 Difference in Gender

Table 3 lists the regression curves calculated for the data described above, and the significance of gender difference was examined by standardized major axis test (SMA) analysis.

The rate of decrease in the number of T-cells, naïve T-cells, and $CD8^+CD28^+$ cells; T-cell proliferation index (TCPI), SIV-5 parameters was slower in females than in males. Further, this difference was statistically significant ($p < 0.05 – 0.003$). The

Table 5 Gender difference in people over 60 years old

	Male over 60 years N=54	Female over 60 years N=48
T-cells (number/mm^3)	1365 ± 53	1395 ± 73
CD4$^+$ T cells (number/mm^3)	910 ± 59	961 ± 55
T-cell proliferation index	1.72 ± 0.11	1.87 ± 0.12
SIV-5 (T-cell immune score)	11.6 ± 0.3	11.7 ± 0.3
CD8$^+$CD28$^+$ (number/mm^3)	181 ± 19	200 ± 20

rate of increase in the number of CD4$^+$ T-cells was greater in females than in males and a statistically significant difference was observed between males and females ($p < 0.005$). In other words, the slower rate of decline or the greater rate of increase in these parameters may indicate that the immunological functions are relatively well preserved in elderly females than in elderly males; this finding may be consistent with the fact that women survive for longer periods than men. Table 5 shows the values of these immunological parameters in elderly males and females over 60 years of age. All parameters show higher values in elderly females than in males; however, the difference is statistically not significant because of the small sample size.

4 Discussion

In 1992, we reported age-related change in subpopulations of lymphocytes in healthy subjects ranging in age from 6 to 102 years (Utsuyama et al. 1992). In the present study, we confirmed most of the results presented in our previous report; i.e., an age-related decrease in CD3$^+$ T-cells, more pronounced decrease in CD8$^+$ T-cells than in CD4$^+$ T-cells, an age-related increase in CD4/CD8 ratio, a decrease in the number of naïve T-cells with a concomitant increase in memory T-cells, a decrease in B-cells and an increase in NK-cells.

In the present study, we examined the proliferative activity of T-cells and confirmed that it gradually declines with advancing age. In addition, we developed a new parameter, T-cell proliferation index (TCPI), which is calculated by using the proliferative activity and the number of T-cells. TCPI was also observed to significantly decrease with age.

It is interesting to note that the rate of decline in the studied parameters differed with gender. The rate of decline in the number of T-cells calculated by the regression curve was -6.150 in males and -2.390 in females. The rate of decline in TCPI was -0.016 in males and -0.008 in females. This gender difference in the T-cells and TCPI vales was statistically significant (Table 3). A relatively gradual decrease in the studied parameters in females than in males may be consistent with the fact that women survive for longer period than men in Japan.

A low number of CD8$^+$CD28- T-cells and high CD4/CD8 ratio are associated with populations that survive until the age of 100 years (Strindhall et al. 2007). Susceptibility to influenza infection in older adults is associated with an increased population of CD8$^+$CD28- T-cells (Xie, McElhaney 2007). In this respect, we confirmed that the number of CD8$^+$CD28$^+$ cells decreased with age and this decrease

was associated with a decrease in the T-cell proliferative response (MTS-OD_{490}); i.e., the rate of decline was significantly slower in females than in males.

The absolute number of total B-lymphocytes increases about 3-fold from the base line in the first year of life and progressively decreases until adult age (Veneri et al. 2007). We further confirmed that the number of B-cells continued to gradually decrease throughout the life and decline was significantly steeper in males than in females.

It is still not clear whether the age-related increase in the prevalence of $CD4^+CD25$(high) regulatory T-cells (TREGs) is responsible for immune dysfunction in the elderly (Dejaco et al. 2006). In this respect, we found that the number of TREGs showed an increasing trend with age.

An age-related increase was observed in the number of NK-cells and this rate of increase was significantly steeper in females than in males. In this respect, Lee et al. (1996) reported that higher percentage of NK-cells in the Asian population than in Caucasian subjects.

Olsson et al. (2000) reported that a decrease in the CD4/CD8 ratio was an important indicator of the immune risk phenotype (IRP). In the present survey, a contrasting feature was observed between $CD4^+$T-cells and $CD8^+$T-cells. The number of $CD4^+$ T-cells was relatively steady level or showed an increasing trend with age, while the number of $CD8^+$ T-cells significantly decreased with age; therefore, the CD4/CD8 ratio showed a distinct age-related increase. Higher percentage and number of $CD8^+$ T-cells and a decreased CD4/CD8 ratio was observed in the Saudi male population compared with Caucasian controls (Shababuddin 1995). Hence, racial difference should be considered in this case.

Anti-CD3 stimulation of T-lymphocytes significantly increased IL-8 production and this increase was more evident in the nonagenarian subjects (Mariani E et al. 2001). Centenarians showed high level of IL-8, indicating that an increased level of IL-8 is related to longevity (Wieczorowska-Tobis et al. 2006). These reports were consistent with the result of the present study indicating an age-related increase in IL-8.

Individuals who are genetically predisposed to produce high level of IL-6 have a reduced capacity to reach the extreme limits of the human lifespan. On the other hand, a high IL-10 producing genotype is observed among centenarians (Caruso C et al. 2004). These results were partly consistent with those of the present study, which showed that both IL-6 and IL-10 decreased with age. In future studies, an adequate sample size should be selected for analysis of cytokine production.

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Age-associated T-cell Clonal Expansions (TCE) in vivo—Implications for Pathogen Resistance

Cellular Immunosenescence – T cells

Janko Nikolich-Žugich and Anna Lang

Contents

Abbreviations

pMHC	peptide MHC complex
RTE	recent thymic emigrants
SPF	specific pathogen-free
TCE	T-cell clonal expansions

Abstract: Age-related T-cell clonal expansions (TCE) are an incompletely understood disturbance in T-cell homeostasis found frequently in old humans and experimental animals. These accumulations of CD8 T-cells have the potential to distort T-cell population balance and reduce T-cell repertoire diversity above and beyond the changes seen in the aging of T-cell pool in the absence of TCE. This chapter discusses our current knowledge of the role of these expansions in health and disease, with a special focus on their influence upon immune defense against infectious diseases.

Keywords: Ageing • Clonal expansions • Homeostasis • Infectious diseases • T-cells

J. Nikolich-Žugich (✉) · A. Lang
Department of Immunobiology and the Arizona Center on Aging
University of Arizona college of Medicine
Tucson, AZ 85719, USA
Phone: +1-520-626-6065
Fax: +1-520-626-2100
E-mail: nikolich@arizona.edu

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1 Introduction

As was extensively discussed in other chapters of this handbook, immunosenescence encompasses a number of diverse age-related cellular and extracellular milieu changes that affect cells and molecules of the immune and inflammatory system. The very definition of immunosenescence, however, operationally includes not only the decline of immunity with age by itself, but also its most important clinical manifestation, the increased susceptibility to infection and decreased immunosurveillance of cancer. Other factors can contribute to the increased exposure to infectious diseases and increased colonization with infectious pathogens (e.g., reduced barrier function of skin and mucosal membranes) with age, and multiple factors certainly strongly contribute to the age-related increase in incidence of cancer. However, it is clear that the inability to mount rapid and vigorous immune defense once an infectious invasion (and, likely, detectable malignant transformation) had occurred lies at the heart of many of the clinical manifestations of immunosenescence. Due to the involvement of numerous other nonimmunological factors in the age-related increase of cancer-related morbidity and mortality, this review will solely deal with infectious diseases.

It has long been known that aging is accompanied by an increase in mortality and morbidity from a number of common respiratory infections such as influenza (20,000–40,000 annual deaths in the USA alone) (Bender 2003; Betts and Treanor 2000; Couch et al. 1986; Glezen and Couch 2003; High 2004; Yoshikawa 2000), pneumococcal pneumonia (Bender 2003; High et al. 2005; Yoshikawa 2000) and RSV (Glezen and Couch 2003; High et al. 2005; Yoshikawa 2000), and urinary infections (Bender 2003; Hazelett et al. 2006). Moreover, this vulnerability extends to dangerous established pathogens such as variola (Hanna 1913) as well as the newly emerging pathogens that disproportionally affect the elderly such as the West Nile virus (Murray et al. 2006) the Severe Acute Respiratory Syndrome-causing Coronavirus (SARS-CoV) (Chan et al. 2007; Leung et al. 2004) and others.

Several types of age-related defects in the immune function can contribute to this increased susceptibility to infection, including defects in innate immunity, antigen uptake, processing and presentation, provision of second and third signals to the adaptive immune system and impaired humoral immunity, all of which are competently covered in other chapters of this handbook. However, T-cells have been known to exhibit some of the most pronounced age-related defects (Miller 1996), and intervention to correct these defects resulted in successful correction of the immune function in a number of cases (Effros et al. 1991; Haynes et al. 2004; Haynes et al. 1999; Messaoudi et al. 2006a). These defects can be grossly divided into cell-autonomous defects, which affect T-cells regardless of age-related or compensatory alterations that affect other components of the immune system and which can be detected in assays where T-cells are the only component of the immune system affected by aging; and age-related changes in the T-cell population balance, which mostly involve the initial loss of naïve T-cells and the compensatory, reactive changes aimed to maintain T-cell homeostasis in the face of this loss.

This chapter will focus upon the latter changes, given that other aspects of T-cell dysfunction will be covered in other chapters of this volume. Moreover, we will discuss the impact of a specific type of age-related T-cell disturbances, T-cell clonal expansions (TCE) (Callahan et al. 1993; Hingorani et al. 1993a; Posnett et al. 1994), upon immune defense and pathogen resistance, highlighting the extent and the limits of our current knowledge, and the tasks and problems that need to be solved before we can fully understand and treat these disturbances.

2 T-cell Homeostasis and Development of T-cell Clonal Expansions (TCE)

The current evidence strongly suggests that the involution of the thymus and the decline in production of new naïve T-cells are the initiating factors behind the generation of at least some TCE (Messaoudi et al. 2006b), whereas latent persistent viral infections may be the perpetrators driving other types of TCE (Pawelec et al. 2004). Moreover, homeostatic mechanisms that are activated as a consequence of naïve T-cell loss may themselves participate in the onset and/or maintenance of TCE (Messaoudi et al. 2006b, 2006c). Therefore, at the risk of being redundant, we will very briefly review thymic T-cell production, involution, latent persistent infections and T-cell homeostasis. For a more detailed review of these topics, the reader is encouraged to read sections of this handbook devoted to thymic involution, as well as the recent volume of Seminars in Immunology devoted to T-cell rejuvenation (Nikolich-Žugich 2007; Zuniga-Pflucker and van den Brink 2007).

2.1 Homeostatic Maintenance of T–cell Subsets

T-cell homeostasis is defined here as maintenance of naïve and memory T-cell pool numbers and diversity and the ability to restore these numbers and diversity following antigenic (Ag) challenge. T-cell homeostasis is regulated by the response of T-cells to environmental trophic and survival signals and by the presence and availability of such signals. The most important and best understood of these signals are the common γ-chain cytokines (most notably IL-7, IL-15 and IL-2) and self-peptide: MHC (pMHC) complexes. The contribution of each of these signals to homeostatic maintenance varies depending on the T-cell subset.

Following maturation and selection in the thymus, new T-cells are released into the periphery as recent thymic emigrants (RTEs) (Scollay et al. 1980). Release of RTEs bearing a variety of randomly rearranged TCRs ensures the diversity of the peripheral T-cell pool. Once released from the thymus, the RTE join the naïve T-cell pool. Naïve T-cells have no preset life spans and are maintained by IL-7 and trophic signals from interaction of their TCR with self-p:MHC complexes (rev. in (Lee and Surh 2005). When these two signals are present, naïve T-cells are believed to be

able to survive indefinitely, based upon the results of serial transfer experiments (Sprent et al. 1991). Murine RTE proliferate faster than naïve peripheral T-cells in the first three weeks after export, perhaps in order to maximize naïve T-cell diversity, before they equilibrate with other naïve T-cells (Berzins et al. 1998). Naïve T-cells display very low levels of spontaneous (or homeostatic) cycling in vivo. Homeostatic cycling is greatly increased in lymphopenia, where T-cells sense a signal, most likely provided by an excess of unused IL-7 and IL-15 (Surh and Sprent 2002). Under lymphopenic conditions T-cells undergo Ag-independent homeostatic proliferative expansion (HPE), in a seeming attempt to fill the empty compartment (Fry and Mackall 2005; Surh et al. 2006). Unlike naïve T-cells, memory T-cells do not require specific p: MHC contact for survival. Instead, their survival is dependent on continued homeostatic proliferation, driven mainly by IL-15, or by IL-7 in the absence of IL-15. Memory cells cycle and self-renew in vivo significantly (up to four times) faster than naïve T-cells and also exhibit faster proliferation during lymphopenia (Surh et al. 2006). It is likely that there may be other, presently unknown pathways regulating T-cell homeostasis, some of which could include energy metabolism regulation (Frauwirth and Thompson 2004).

The above described homeostatic mechanisms function to maintain a balanced and diverse T-cell pool. Over lifetime this means regulating the process of Ag-driven expansion of naïve T-cells, their contraction, and selection and maintenance of memory T-cells. The role of the homeostatic mechanisms is to balance the composition of the T-cell pool so that it contains both naïve precursors with diverse TCRs, as well as Ag-experienced memory T-cells, as both of these subsets are crucial for the health of the host. The homeostatic forces work very efficiently in adult mice housed under specific pathogen free (SPF) conditions, as evidenced by remarkably similar size and diversity of the T-cell pool among individual mice of the same strain. However, maintenance of homeostasis becomes more complicated in the face of constant encounters with new acute pathogens, long-term interactions with persistent pathogens and the aging-associated defects, all of which are discussed below.

2.2 Disruption of T-cell Homeostasis in Ageing

Thymic involution begins soon after birth in humans and quickly after puberty in mice, which results in decreased RTE output (Haynes et al. 2000; Hirokawa and Utsuyama 1984). Thus, 22-mo-old mice receive less than 10% of RTE compared to young adult mice (Hale et al. 2006; Heng et al. 2005). Even in old age the thymus continues to produce RTE proportionally to its overall cellularity, but as the cellularity itself decreases, so does the output (Gruver et al. 2007; Hale et al. 2006). The cause of thymic involution is discussed in more detail elsewhere in this volume. From the standpoint of this chapter, thymic involution presents a challenge for the homeostatic mechanisms, which strive to maintain the size and diversity of the peripheral T-cell pool in the face of decreased influx of diverse new T-cells.

Despite the fact that thymus involution begins early in life, it is only in old age that homeostatic mechanisms falter and allow dysbalance amongst T-cell subsets.

A marked difference between the adult and old lymphocyte T-cell compartment is an age-related decrease in representation of naïve phenotype T-cells and concomitant increase in frequency and numbers of memory phenotype T-cells. The exact mechanisms leading to this population shift were not formally dissected, but are believed to likely involve a combination of 1) decrease in naïve T-cell production, 2) their conversion into effector or memory cells as a result of encounters with pathogens, and 3) changes in the environment, including the availability of homeostatic cytokines (IL-7, IL-15, IL-2). For example, IL-2 production by CD4 T-cells is decreased in old mice(Gillis et al. 1981; Miller and Stutman 1981; Thoman and Weigle 1981). Less is known about age-related changes in IL-7 or IL-15 levels or the expression and function of their receptors on different T-cell subsets. In addition, the naïve T-cell pool could be indirectly affected by a growing pool of memory T-cells that may compete with naïve T-cells. Considering that there is some overlap in the use of survival and maintenance cytokines by these two pools, particularly in case of IL-7(Fry, Mackall 2005; Tan et al. 2002), it is possible that the two are not always independently regulated, particularly in aging where there is many fewer naïve T-cells. Thus, if naïve T-cells continue to decrease in number, this may lead to an excess of survival and maintenance cytokines which normally would have been consumed by naïve T-cells. This could trigger homeostatic proliferative expansion (HPE) of the remaining naïve T-cells and drive their conversion to memory-phenotype. This was demonstrated in mice under lymphopenic conditions (Cho et al. 2000; Goldrath et al. 2000), and strongly suggestive results were also obtained in aging monkeys (Cicin-Sain et al. 2007) and humans (Naylor et al. 2005).

2.3 T-cell Clonal Expansions (TCE)

One of the hallmarks of immune aging is loss of TCR repertoire diversity (rev. in (Nikolich-Žugich 2005)), due in large part to the dominance of memory T-cells over the naïve ones. However, on top of that reduction, the CD8 T-cell compartment often shows additional loss of diversity, in the form of large, often clonal expansions of T-cells bearing the same TCR, named T-cell clonal expansions (TCE) (Callahan et al. 1993; Hingorani et al. 1993b; Posnett et al. 1994). Development of TCEs has been documented across mammalian species, including rodents, nonhuman primates, and humans, with fractions between 30 and 60% of individuals surveyed exhibiting one or more age-associated TCE (rev. in (Nikolich-Žugich, Messaoudi 2005). More on the biology of TCE can be found in the excellent review by Clambey and Marrack elsewhere in this book. However, for the purpose of this chapter, it is most pertinent to classify TCE into at least two types with respect to the mechanism of their generation and/or maintenance. Large Ag-independent TCE (AI-TCE) are thought to arise and/or be maintained independently of antigenic stimulation, due to age-

related changes in perceiving homeostatic signals. This is based upon: (i) activation marker expression on these cells, which dominantly exhibit central memory phenotype, with no evidence of recent or repeated antigen-driven activation (Callahan et al. 1993; Ku et al. 2001; Messaoudi et al. 2006c); (ii) cytokine receptor, specifically IL-7R and IL-15R, expression, which is higher on these cells compared to other memory or naïve T-cells (Messaoudi et al. 2006c); (iii) the ability of these cells to proliferate upon adoptive transfer (Ku et al. 2001), with a constant rate regardless of whether the recipient is lymphopenic or not (Messaoudi et al. 2006c); and (iv) the ability of manipulations that induce lymphopenia to increase the incidence and accelerate the onset of development of AI-TCE (Messaoudi et al. 2006b). While these results have been obtained in mice, there is evidence that similar fundamental principles are at work in primates, including humans (Cicin-Sain et al. 2007; Naylor et al. 2005). In contrast, TCE that have general characteristics consistent with the response to antigen, also called Ag-reactive TCE (AR-TCE), were linked to latent persistent herpesviral infections in mice (Holtappels et al. 2000; Karrer et al. 2003; Podlech et al. 2000) and humans (Almanzar et al. 2005; Fletcher et al. 2005; Ouyang et al. 2003c; Pawelec et al. 2004). Broad discussion of these virus-related abnormalities is also presented in other chapters of this handbook.

TCE can occupy up to 90% of the total murine and up to 50% of the human memory CD8 T-cell pool. TCE themselves are not malignant and do not affect the overall size of the CD8 T-cell pool (there is no increase in total T-cell numbers in individuals carrying TCE). However, TCE do disturb T-cell homeostasis and diversity (Callahan et al. 1993; LeMaoult et al. 2000; Posnett et al. 1994) and a drastic disturbance of this type can be expected to impair the ability to mount T-cell responses. While T-cell responses are plastic, with a significant reserve that allows T-cells to respond to pathogens despite loss of much of the repertoire, this plasticity is not unlimited (rev. in (Nikolich-Žugich et al. 2004). However, we still do not have precise quantitative understanding of limits of T-cell diversity necessary to mount protective responses against pathogenic challenge, an issue highly relevant from the standpoint of evaluating the impact of TCE upon immune defense.

3 Impact of TCE on Pathogen Resistance—the Mouse Model

The most important question related to the presence of TCE is related to their impact upon the health of the organism. One could envision several possibilities in that regard. First, TCE could be neutral and not impact the overall health or the immune defense of the old organism. While this possibility is intellectually unexciting, it is likely that many TCE coexist with the state of health based on their high incidence in asymptomatic individuals (Hingorani et al. 1993b; Posnett et al. 1994). Indeed, it is likely that a TCE needs to grow to a certain size before it becomes a problem for its bearer. Second, TCE could affect other components of the organism, without impacting immune defense. While this is possible, this scenario had not been

documented so far and will not be further discussed here. Third, TCE could have an active effect, whereby they would secrete cytokines and other short-acting mediators that could alter the function of other components of the immune (and other) systems in the body. This would be akin to the functional shift seen in replicatively senescent fibroblasts, which upon cessation of replication drastically change their secretory properties and have the potential to alter extracellular matrix, neovascularization and other microenvironmental properties (rev. in (Campisi 2002). At the present, there is some evidence in support of this possibility (Ortiz-Suarez and Miller 2002; Ortiz-Suarez and Miller 2003), but more precise studies at the level of isolated, highly purified TCE are needed. Moreover, the impact of the observed changes upon pathogen resistance remains untested.

Finally, the role of TCE could be passive, but nevertheless negative. Under that scenario, which was invoked by immunologists before (Callahan et al. 1993; Hingorani et al. 1993a; Posnett 1994 #1976), and which will be discussed in more detail as it currently appears the most likely, these accumulating T-cell clones would constrict the repertoire and reduce the useful T-cell repertoire that defends us against new infection. Mechanistically, this would most likely occur by these cells gaining a survival/maintenance advantage over other T-cells in the body. The fact that TCE which occur spontaneously in SPF mice express high levels of IL-7Rα and IL-2/15Rβ (Messaoudi et al. 2006c) is consistent with the possibility that TCE operate as IL-7 and/or IL-15 "cytokine sinks", taking them slowly away from other T-cells. Consistent with that, we (Lang et al. submitted) and others (Ely et al., 2007) have recently found that often TCE can arise from the pool of cells that respond(ed) to prior acute or latent infection. Of interest, once these cells begin to significantly expand in old age, they tend to acquire high levels of IL-7 and IL-15 receptors (Lang et al. submitted), raising the possibility that the "cytokine sink" may be the unifying mechanism by which both "spontaneous" and antigen-specific large TCE constrict the remainder of useful T-cell repertoire. In fact, it is likely that the "spontaneous" TCE designation simply covers up the fact that we don't know the original antigen that was recognized by these cells, and that may be irrelevant if indeed these cells primarily respond to cytokines once they become TCE.

In order for a TCE to have a demonstrably negative effect upon immune defense via TCR repertoire constriction, such a TCE needs to sufficiently erode the numbers and diversity of other T cells needed to respond to a new pathogen. Numerous studies have shown that manipulations which take away up to half or more of TCR diversity are reasonably compatible with T-cell responsiveness (rev. in (Nikolich-Žugich et al. 2004). However, in other models losses of this or greater magnitude have been shown to impair responsiveness to certain antigens (rev in. (Nikolich-Žugich et al. 2004) and references therein). In terms of the impact of TCE upon the residual diversity of aged naïve T- cells in relationship to immune defense against infectious diseases, it is important to consider the overall diversity and overall numbers of T-cells involved in a typical response to a pathogen. Exciting new studies with direct measuring of precursor T-cell frequencies concur that on the average a hundred, and in some cases as few as 15-20 CD4 or CD8 T-cells may

be responding to a single epitope (Badovinac et al. 2007; Moon et al. 2007). Even if this is an underestimation, reducing that number by 90%, or even by half, due to the presence of a TCE, certainly has the potential to diminish and cripple the response to epitopes where few T-cell precursors exist. This low responsiveness would be further compounded by an already diminished overall reserve of naïve T-cells in aging, as well as by the blunted T-cell signaling (Tamir et al. 2000). On the other hand, most pathogens present multiple epitopes to the immune system, and even if one accounts for immunodominance, usually a handful of epitopes are available for T-cell stimulation. Moreover, in many cases other arms of the immune system will synergize to provide protection even if T-cell responses are diminished. Thus, for a TCE to impact pathogen resistance, T-cells have to provide primary and nonredundant protection against that pathogen, the pathogen should have few, rather than many, immunodominant and protective epitopes and frequency of T-cells specific for these epitopes should be low. In the one case where the impact of TCE upon immune defense was tested (Messaoudi et al. 2004), most, if not all, of the above conditions were met. In that study, resistance to herpes simplex virus (HSV-1) was studied in B6 mice, where an octamer derived from the glycoprotein B accounts for > 90% of the total CD8 T-cell response (Dyall et al. 2000; Messaoudi 2001 #1644; Wallace et al. 1999). Moreover, the response itself is highly restricted with regard to TCRV region utilization (with Vβ10 and 8 contributing >80% of the response (Cose et al. 1995)). Old animals with and without TCE were challenged with HSV and magnitude and functional characteristics of the response measured. It was found that TCE could impair the generation of productive responses in a selective manner. So, when an animal contained a large TCE which expressed Vβ10 and 8, it was unable to mount a response to HSV gB, whereas TCE expressing other TCR Vβ segments did not impair responsiveness beyond the reduction seen due to age in a littermate control group (Messaoudi et al. 2004). These results were somewhat puzzling and suggested that TCE preferentially competed out against the T-cells bearing the same TCRVβ segment. This could be explained, for example, if TCRVβ residues conserved within the Vβ family but differing between Vβ families (e.g. CDR1 & 2 and "framework" parts of CDR3) were important in contacting self-pMHC complexes in the course of trophic interactions needed for T-cell maintenance, so that a TCE would compete out naïve T-cells of the same TCRVβ family. Such a mechanism remains to be substantiated. Nevertheless, the above study (Messaoudi et al. 2004) does show that TCE can potentially impair protective immunity.

While the above experiments were performed with spontaneously arising TCE, which were most likely AI-TCE, there is no reason to believe that a similar situation may not exist with AR-TCE as well. Our group is in the process of testing this possibility. Another unaddressed question relates to the impact of TCE upon memory responses. Memory T-cells are more difficult to compete out than naïve T-cells, possibly due to their ability for self-renewal and relative resistance to apoptosis. Perhaps the most pertinent question is whether TCE can affect the response to latent and/or chronic persistent pathogens, where a large fraction of the immune system is periodically or continuously stimulated by these pathogens. At the present, this issue remains unresolved.

4 Impact of TCE on Pathogen Resistance—Evidence from Humans

In reviewing the known impact of TCEs on pathogen resistance, one needs to distinguish between two parameters: 1) correlation of presence of TCE with presence of other immunological factors known to impair immune responses, and 2) direct evidence for impact of TCE on pathogen resistance. The occurrence of TCEs has been well documented in patients and in a variety of animal models, so we shall first review that scenario. One should bear in mind, however, that it is often difficult to distinguish the specific effect of TCE from the effects of old age-associated defects in antipathogen immunity, since in most cases TCEs are detected only in advanced age. It is therefore most appropriate to evaluate TCE as a superimposing, possibly aggravating factor that may, or may not, further impair protective immunity in an already suboptimal setting of an old organism.

Some TCEs have known antigenic specificity. Two types of conclusions on the effects of these TCEs on pathogen resistance can be drawn: 1) effect upon resistance to the pathogen the TCE is specific for, and 2) effect upon resistance to unrelated pathogens. In humans, the most commonly documented cases of TCEs of known specificity involve memory CD8 T-cells specific for CMV (rev. in (Pawelec et al. 2004)) and, to a lesser extent, EBV (Ouyang et al. 2003b). Original studies documented the presence of $CD28^{-}$ $CD8^{+}$ TCEs in elderly patients (Hingorani et al. 1993b; Posnett et al. 1994). With the advent of tetramers and intracellular cytokine staining techniques that allowed enumeration of Ag-specific T-cells, it was shown that the CD28- CD8 T-cell expansions were frequently specific for CMV and were clonal or oligoclonal in nature (Ouyang et al. 2002). Moreover, longitudinal studies in the Swedish elderly cohorts concluded that CMV seropositivity, together with an array of additional immune characteristics such as the inverted CD4:CD8 ratio and poor proliferative responses of T-cells to mitogens, constitute an immune risk phenotype (IRP, discussed in detail elsewhere in this book) (Wikby et al. 2005), which predicted mortality within 2 years in octogenerians of the Swedish cohort (Hadrup et al. 2006). It will be important to reproduce these results in genetically diverse populations of the elderly, particularly in light of early reports that the elderly from West Sicily may not show the same effect (Colonna-Romano et al. 2007). Moreover, it is not clear exactly how the presence of CMV-specific TCE might affect pathogen resistance, in isolation from the other IRP-associated defects, highlighting one of the problems inherent to the otherwise highly informative human longitudinal studies.

At the present, there is some evidence that CMV-specific T-cells may themselves be compromised as a direct result of development of TCE. Several studies demonstrated accumulation of dysfunctional CMV-specific memory CD8 T-cells in the elderly (Ouyang et al. 2003a; Ouyang et al. 2003c; Ouyang et al. 2004). In addition, the large CMV-specific memory cell population expressed a marker of replicative senescence, KLRG-1, and its expression correlated with decreased production of IFNγ upon antigenic stimulation (Ouyang et al. 2004). The key question is whether this leads to inability to mount an adequate functional response to viral reactivation, permitting viral replication above the subclinical level normally associated with

CMV seropositivity. In that regard, one study (Stowe et al. 2007) demonstrated the presence of CMV and EBV DNA in urine (CMV) and blood (EBV) of elderly patients, as opposed to the seropositive adults, implying some loss of control of viral reactivation in the elderly. Consistent with that explanation, these authors also found elevated expression of lytic and latent EBV genes in blood of elderly but not adult seropositive patients (Stowe et al. 2007). It is possible that accumulation of dysfunctional CMV- or EBV-specific TCE, which were unable to control the virus, may be the reason for increase in viral reactivation in aging. However, in that study, the elderly actually had an elevated frequency of IFNγ-producing CMV- and EBV-specific memory CD8 T-cells, making the hypothesis unlikely. Moreover, CMV-mediated disease does not seem to be associated with aging in the absence of iatrogenic or acquired immune suppression, suggesting that a manifest loss of CMV control does not occur in the elderly. Further studies are needed to decisively address the role of accumulation of dysfunctional CMV-specific TCEs on the persistent latent Herpes virus control in old age.

A separate issue is whether CMV-specific TCE affect immunity to other infections in humans, and how. There is some evidence that presence of CMV-specific TCE is associated with lower frequency of memory CD8 T-cells specific for coresident EBV infection (Khan et al. 2004). This study did not examine whether control of latent EBV in patients with large CMV-specific TCE is impaired. While one could speculate that the T-cell response, and therefore immunity to EBV will be compromised in patients with large CMV-specific TCE similar to the results seen in mice with the effect of spontaneous TCE upon HSV immunity (Messaoudi et al. 2004), the mechanism by which these TCE affect the size of the EBV memory CD8 T-cell pool is currently unknown.

Since many of the TCEs identified in humans are specific for CMV, it was proposed that CMV is the main driver behind generation of TCEs (Pawelec et al. 2004). While this may be the case, evidence from murine studies suggests that virus-specific TCE can also develop independently from ongoing antigenic stimulation. Ely et al. (Ely et al. 2007) detected presence of TCE specific for Sendai virus and flu in old mice that had been infected as adults. Similarly, we have found that old mice infected with WNV at a young age developed expansions of virus-specific memory CD8 T-cells in old age (A Lang et al. submitted). In a different infection model, we found that following localized (ocular) HSV-1 infection, mice develop expansions of HSV-specific memory CD8 T-cells once they reach old age. This process was unlikely to be caused by viral reactivation, as mice treated continuously with antiviral drugs also developed these age-associated T-cell expansions. At present, only a small number of these antigen-independent age-associated expansions were confirmed to be clonal, with oligoclonality being seen more often (A Lang et al. submitted). Unlike is the case with CMV-specific TCEs, the T-cell expansions that developed independently from ongoing antigenic stimulation were fully functional, showing excellent correlation of percentage of tetramer$^+$ and IFNγ$^+$ cells (A Lang et al. submitted). Therefore, it is not likely that development of TCEs by this mechanism will affect immunity to the cognate pathogen. Additional studies will be required to determine whether TCEs can develop from

preexisting memory CD8 T-cells specific for nonpersisting pathogens in elderly humans, as they do in old mice.

Are these TCE impairing productive immunity in humans? Of interest, the number of influenza-specific memory CD8 T-cells was shown to decline with age in humans (Goronzy et al. 2001). This phenomenon was independent of the patients' CMV status. In another study of success of flu vaccination in CMV-seropositive patients, CMV seropositivity correlated with impaired response to vaccination (Saurwein-Teissl et al. 2002). However, the authors did not delineate whether this correlates best to the presence of TCE, to the overall decrease in number of naïve cells or to proliferative/replicative senescence, and, as with most clinical studies, the mechanism responsible for this outcome has not been resolved. Therefore, the presence of TCE could be one of the useful biomarkers predicting poor outcome of flu vaccination (Goronzy et al. 2001; Saurwein-Teissl et al. 2002), or perhaps even general immunological vulnerability, but that requires further and rigorous verification in larger and heterogeneous populations of human subjects.

5 Concluding Remarks, Challenges and Questions

It follows from the above discussion that much remains to be learned about the biology of TCE and their precise impact upon resistance to infectious diseases. Drawing generalized conclusions about the impact of TCEs on pathogen resistance from the available data is often difficult, since they come from a number of different experimental models. At present we do not know how closely the mechanisms of generation of TCEs and their subsequent effects on pathogen resistance compare between them. However, the models and the reagents that are currently available provide good tools to systematically address the questions that still remain regarding the impact of TCEs on immunity. In particular, new quantitative tools are becoming available allowing us to precisely dissect the breadth and the reserve of T-cell receptor repertoire and the size of precursor populations specific for immunodominant epitopes of various pathogens, and that should allow us to quantitatively evaluate to what extent is TCR repertoire constricted by different types of TCE, and to determine what type of intervention (many of which are now in clinical trials (Zuniga-Pflucker, van den Brink 2007)) could be applied in individual situations.

Overall, the most important practical issues related to TCE and the infectious diseases of the elderly are:

1. Which groups of elderly are at an increased risk of infection and which are not? Are TCE a risk factor in that regard?
2. For those groups that are at risk, can they be helped with the existing vaccines or do they need alternate ways of immunostimulation? Can TCE be removed or shrunken?
3. If immunostimulation is to be attempted in a targeted manner, which modes of immunostimulation are the most efficacious? Different vaccination regimens, additional costimulation or cytokine treatments?

4. For those where immunostimulation may be insufficient, is T-cell rejuvenation the best option?

Answering these questions will undoubtedly be rewarding for scientists and physicians,as well as to the growing populations of elderly around the world.

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T-cell Cycle and Immunosenescence: Role of Aging in the T-cell Proliferative Behaviour and Status Quo Maintenance

Jacek M. Witkowski

Contents

Organismal aging is affecting the performance of the immune system of mammals (including human one, being the topic of this chapter), and usually is associated with decreased ability to built adequate immune response to new and even cognate antigenic challenges on one side, and with reported increased frequency of autoimmune reactivity against own antigens [34, 61, 64] (but see the chapter by Ewa Bryl and JMW in this volume). Common manifestations of this immunological impairment are thus increased susceptibility (and more difficult curability) of infectious diseases (which in the old age become one of the most important killers despite the achievements of modern "western" medicine), as well as increased frequencies of at least certain malignancies.

The pool of T-lymphocytes, getting their name from its intrathymic period of maturation and selection after exiting the bone marrow and prior to settling in the peripheral lymphatic organs, is a variable, multifunctional and multi-phenotype group of not-so-similar cells. There is—of course—the basic subdivision into the "T-helper" or $CD4^+$ and "T cytotoxic/suppressor" $CD8^+$ lymphocytes, but this is by far a simplification. Thus, within each of the abovementioned, one would encounter first the subpopulations differing in their "life history" prior to the moment of analysis. Among them, the cells that are still "fresh" from the thymus, or had never yet encountered the antigenic epitope for which their T-cell receptors or TCRs were selected, are rightly called the naïve or virgin T-lymphocytes, while those that are a result of such an encounter would be further subdivided into the—relatively short-lived—effector lymphocytes and the supposedly long-lived memory cells (but see below on the lifespan of naïve and memory T-cells). Within the latter, a further subdivision exists that allows the distinction of so called central and effector memory cells. These subpopulations can be relatively easily detected and quantified with the use of monoclonal antibodies recognizing their specific antigens (e.g. CD45RA, CD45R0, CD62L, CCR7 and many others, as described in all current immunology

J. M. Witkowski (✉)
Department of Pathophysiology
Medical University of Gdansk
Poland

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textbooks) and flow cytometry, in the samples of peripheral blood, and—when an animal (usually mouse) model is studied, also in other lymphatic organs and bone marrow.

All these cell types function with one major goal: to survey the organism in search of alien moieties that are or may become damaging to the integrity of the organism, and to develop specific ways of their neutralization and elimination (called adoptive immunity), regardless from their origin, which may obviously be extra- and intra-organismal. To achieve this goal, the T-lymphocytes must properly **interact with each other** within the general, and, as sketched above, already complex "family", by means of either direct contact or secreted mediators (cytokines). The scheme of these interactions is depicted in Fig. 1. Their function towards this goal is not standing alone; contrarily, in order to perform adequately (i.e., to eliminate or otherwise neutralize the alien antigen or cell) T-lymphocytes must interact with, influence and be influenced by other cell types, including the broad family of antigen-presenting cells (professional APCs, requiring the MHC (or HLA) Class II to interact with the $CD4^+$ cells) on one side, and the two remaining groups of lymphocytes—the NK- and B-cells—on the other. One has to remember however, that the above does NOT constitute all contacts and interactions of the T-cells. Thus, practically every cell may influence the behavior of $CD8^+$ lymphocytes via its HLA Class I molecules and epitopes anchored onto them; cells belonging to the players in the inflammatory process (especially, but not exclusively, all forms of macrophages) would affect the T-cells by secreting the "pro-inflammatory" cytokines (e.g., IL-1, IL-6 or TNF) that are known to trigger specific receptors on these cells. Finally, T-lymphocytes contain receptors (and relevant intracellular signalling pathways) able to react to the plethora of other biologically important mediators that may appear in the organism under stress, exercise, injury and on many other instances; these would include first of all neuromediators generated by the central nervous system and hormones (Fig. 1). Through these integrative systems of the organism the lymphocytes get knowledge about its status and about external factors that might require their activity. Any and all of these interactions might be (and, according to the current knowledge mostly are) affected by the aging process.

Now, the effectiveness of an immune response, understood as the quickness and completeness of the neutralization/removal of potentially dangerous antigen is therefore **dependent on the two major, related factors. The first** is the availability of adequate numbers of the cells that can react to the antigenic challenge. They would have appropriate, broad repertoire of the TCR/CD3 complexes and accessory molecules (including first of all the CD4 or CD8 and CD28 in right numbers on their surface, and the intracellular machinery of signal transduction, protein synthesis, DNA replication, cell division etc. in good working order. **The second** stems from the first and is the ability of a T-cell population to temporarily increase numbers of effector T-cells, whose role is to neutralize the invading antigen directly or with the help of cytokine-driven NK killer cells or antibody-producing B-lymphocytes, and which should then quickly get stopped by regulatory/suppressive T-cells and ultimately disappear by the Activation-Induced Cell Death (AICD) being a form of apoptosis (see the chapter by Ewa Sikora in this volume). This second

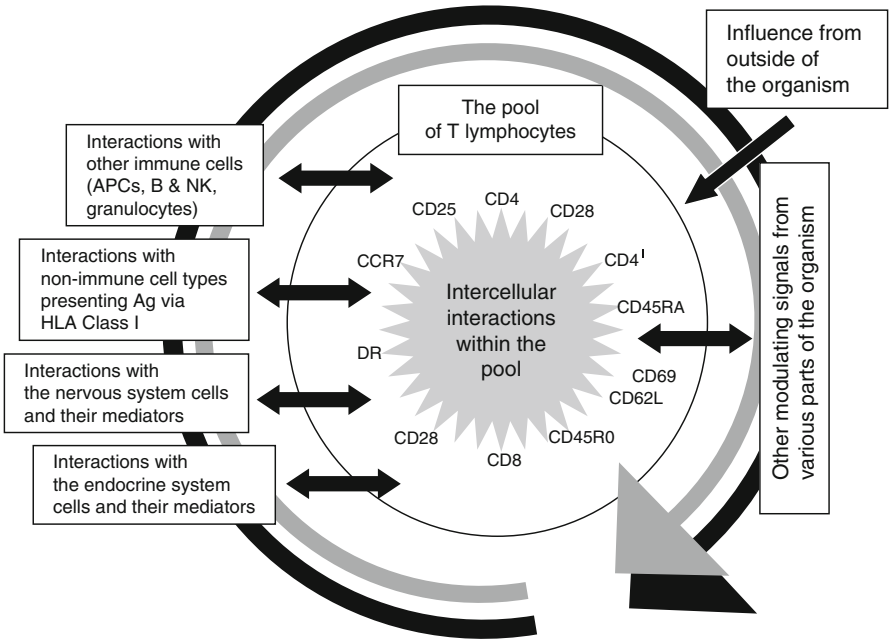

Fig. 1 The idea of T-cells' pool interactions within the broader organismal network, requiring proper numbers and functionalities of all members

utmost important factor ascertaining the optimal immune response is therefore the process, called **T-cell proliferation**.

The above is of course the basic tenet of current immunology. However, what perspires as important for our consideration of aging-related changes in the performance of the immune system, is the need to maintain the right **proportions and numbers** of all of the abovementioned cellular (sub)populations in order to keep the whole system optimally effective (please mark "optimally", which does not mean "maximally" and might mean the difference between the immunity and the autoimmunity). As mentioned above, direct intercellular contacts and humoral signals form the two ways various cells of the immune system communicate with each other and with other cell types. For these "means of communication" to be optimally effective and lead to the goal being the antigen neutralization, the communicating cells have to be in proximity to each other and in adequate numbers, which is maintained by effective proliferation (multiplication) of various T-cell subpopulations responding to an antigenic challenge. It is commonly accepted that both the proportions as well as absolute numbers of various subpopulations of the human T-cell pool change with advancing age which may be at least one of the reasons for decreased overall performance of the system in the elderly.

Thus, T-lymphocyte pool appears as a component of a very complex, dynamic, (and by far not fully understood) web or network of interactions, where the signals conveying information may be either direct intercellular contacts or cellular

(secreted) molecules. Each and every component of this network may undergo aging- and/or pathology-associated changes, affecting its functions and—among other—its interactions with the immune system and with the T-cells in particular. It is a very hard task to understand, how in fact aging *"as such"* is affecting the (T) cells of the immune system (example: a whatever subpopulation of T-cells drawn from an old organism might be absolutely normal and do not functionally differ from the same population drawn from a young organism; however, at the moment of sampling, the nervous (or say, hormonal) system in the old organism could have failed to secrete some mediator or hormone and this lack would affect the T-cell under study leading to an observed different reactivity, when compared with these from a young individual). Therefore in practically all current studies such broad analysis of multivariable status of organisms, from which the immune cells are drawn, is not performed and it is assumed that whatever "extra-T-cell" influences may affect the T-cells of an old individual they would integrate and be relatively similar through the healthy elderly cohort (yet different from the healthy young cohort, as a matter of course). Thus, the result of any test comparing the immune cells' function in the healthy young and elderly bear the burden (and thus—doubts) of our lack of general knowledge about the individual's status preceding the experiment.

While trying to understand the complexity of the T-cell system as a part of the (more general) immune system, its interactions and interrelations (both within the system and with the other ones (Fig. 1)) and, especially, any changes in its function related to advancing age and the process of aging, one has to be very aware of a basic difference between the immune systems of human beings and of model laboratory animals. The latter, usually germ-free or at least "specific pathogen-free" mice, have their immune systems all but dormant until the experimenter challenges them in vivo or in vitro (with possible exception of newly transformed neoplasm cells, that may form an unpredicted source of antigens even under such conditions). On the other hand, our own immune cells are not only on constant alert, but, in fact, constantly in-fight (although not all of them at the same time, of course!); our environment (the air we breathe, the foods and drinks, other members of our species, our pets and farm animals etc.) is full of antigens, both those already known to the immune (memory) cells and the new ones, challenging the naïve T-cell pool.

Within the abovementioned network pervading the organism, the T-cells will dwell only in certain locations or microenvironments, providing for them the relevant survival and sometimes mitogenic signals; these locations can be collectively called the "T-cell niche" (Fig. 2), even if it is already known that different T-cell subpopulations would require different sets of these survival-and-proliferative signals and thus will rather live in a few partially overlapping "niches". A very good example here is the memory T-cell niche, which had recently been shown to be defined by the ligands belonging to the TNF family and in fact differentiating between the $CD8^+$ memory and $CD4^+$ memory "subniches" [74].

Thus, the T-cell niche (being, in fact, a sum of all "T-cell subpopulations' niches") in a human organism is never static. Both the proportions and the absolute numbers of various T-cell (sub)populations are homeostatically maintained throughout most of our adult life, in order to keep this niche (consisting of the T-cell compartments in

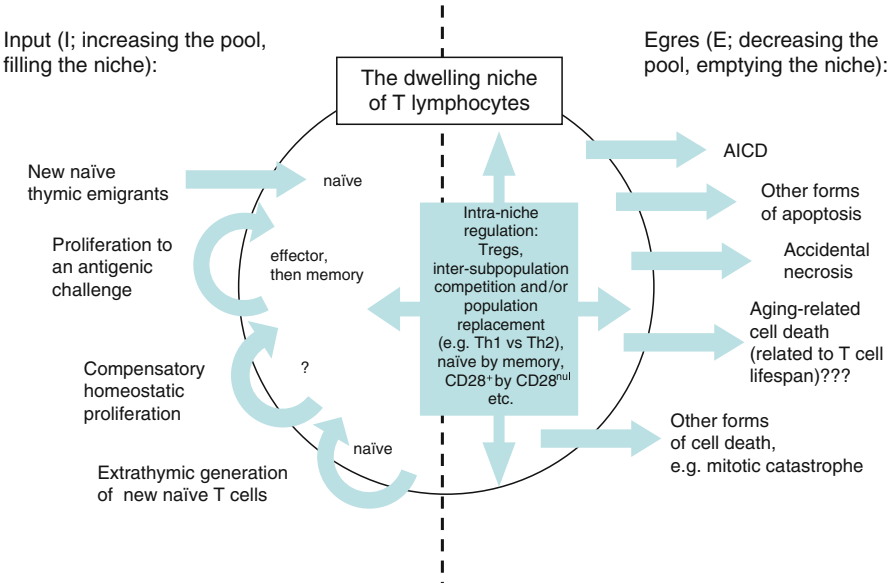

Fig. 2 The idea of T-cell niche homeostasis; I = E

the lymphatic organs, bone marrow and circulating lymphocytes, although the latter can also be considered a "common sink" or corridor for all of the relevant "niches", where members of different ones may meet and interact) relatively constant in volume, yet ready to respond to any antigenic challenge with enough effectiveness to keep the organism healthy (or, at least, to ascertain its survival when confronted by a pathogen). These **homeostatic regulators (homeostats)** consist of the mechanisms leading on one hand to fast and concerted accumulation of all the required effector cells (the "input" side in the Figs. 2 and 3), but containing various ways the T-cell may die (apoptosis, necrosis etc., the "egres" side in the Figs. 2 and 3), including the production of regulatory T-cells with one or another type of suppressive activity against their activated sisters on the other (see below and the chapters by K Hirokawa and by P Moss in this volume for more detail on T-cell homeostasis and regulatory T-cells respectively).

One of the most important questions for our understanding what happens with our T-cells when we age was interesting gerontologists for many years and it is still not answered yet. Why—knowing that our immune system is impaired and generally loosing its functionality when we age—we do not observe major decrease in its volume—in the peripheral blood lymphocytosis for example, or in the palpable volume of the lymph nodes in the old individuals? In other words: what keeps the T-cell niche in an active equilibrium over our young and middle age, i.e., what homeostats play the major role(s) in the process, and how they change when we get old? Let us consider the general situation in the T-cell niche (Fig. 2.). The mechanisms increasing the volume of the niche(s) (the "input" side in the Fig. 2.) can be divided into intrinsic and extrinsic to the niche-dwelling cells itself. Those

intrinsic to the in-niche T-cells themselves will mostly depend on the antigenic (or, in vitro—also mitogenic) challenge. They will consist of: **ability to recognize the stimulatory signal** (an antigen, in vitro also mitogens) from environmental noise, which requires proper diversity and numbers of the TCR/CD3 complexes, **ability to distinguish the signal as "requiring response"** which requires proper MHC/HLA context, availability of other (costimulator) molecules (especially CD28) interacting with the antigen-presenting cells, adequate numbers (surface densities) of these, and the **ability to properly respond to incoming signals** which requires functional signal transduction mechanisms (starting from the proper numbers of relevant T-cell surface receptors), functional gene activation and transcription machinery, functional protein synthesis apparatus. Within this proper response lays, of course, the a**bility to divide** (proliferate, i.e., undergo productive mitosis, leading to the generation of viable daughter (effector) cells), which requires adequately functional cellular machinery directly involved in the processes of error-free DNA replication, and in its separation into newly formed nuclei of the daughter cells.

The mechanisms **extrinsic to the in-niche T-lymphocytes** would contain **influx of new, naïve T**-cells generated in the thymus or extra-thymically, **T-cell survival signals,** which may be generated in the niche or outside (these include IL-2 and other growth factors) and, possibly, also the hypothetical and currently mostly unknown **factors that govern the development and size of the microenvironment** creating the niche stroma. One has also to bear in mind the postulated homeostatic proliferation of the T-cells—one that supposedly provides new naïve T-cells even after the cessation of thymic lymphopoiesis; it can probably also increase or at least sustain the numbers of the memory cells (possibly *ex definitione* also without antigenic stimulation) and thus would get more and more importance with advancing age (see below).

Considering the mechanisms decreasing the volume of the niche(s) or the eliminators of "surplus" (or temporary surplus) T-cells (the "egres" side in the Fig. 2), one would have to list first those intrinsic to the activation process, i.e., any form of Activation Induced Cell Death (AICD). This is a major safety valve against uncontrolled overproduction or protracted dwelling of activated T-lymphocytes in the niche, which could—and sometimes does—result in either autoimmunity or transformation into leukaemic growth, that eliminates practically all no-more-necessary effectors. Apparently, the AICD occurs only or mostly at the early G1A phase of the cell cycle [43]. Other forms of apoptosis will constitute another negative homeostat—like the one related to lack of growth- or survival-promoting factors, or that induced by irreparable (or not repaired soon enough) DNA damage. These would not be limited to any specific phase of the cell cycle.

Another somewhat similar way of elimination of the T-cells will be the **mitotic catastrophe**—when all the signals and processes are in order until the moment of mitosis, where "something" goes wrong and proper separation of genetic material does not occur; however, in case of normal human lymphocytes, this way of cellular dying is not yet well understood or even proven [82].

The T-cell (similarly to other 300+ cell types of our of organism) my also die by other means, not directly related to their physiological function. First of all, they

may die by **accidental necrosis**—when the intracellular compensatory (homeostatic) mechanisms fail when confronted with—for example—a metabolic toxin, lack of oxygen, or (admittedly very unlike for lymphocytes) mechanical damage.

And finally, what should be of an utmost interest considering the topic of this chapter and the entire book, it is possible that **T-cells die "of old age"** i.e., because they had aged so much that their intracellular homeostats cannot support life anymore. This last possibility bears with it another question, that about the actual lifespan of human lymphocytes. Are they short-lived (on the scale of days) and rapidly replaced? Are they long-lived and—when not confronted with an antigenic challenge—is their lifespan comparable to that of the organisms (i.e., measured in many years for human beings)? This question, obviously of an utmost importance for understanding the balance within the T-cell niche, is not so easy to answer. In order to know the actual (or maximal) lifespan of any T-cells' subpopulation, one would have to mark it somehow at the beginning of the individuals' life and then observe how long these marked cells would stay present in the organism under study. In fact, such analysis has been performed both for mice and for humans. In the former, it is possible to draw and isolate the lymphocytes, mark them with a stable fluorescent tag (for example the carboxyfluorescein diacetate succinimidyl ester (CFSE)), reinject in the animal and then seek the fluorescent cells in the blood or lymphatic organ of the animal after at least many months, which correspond to a substantial portion of the animals' life [46]. The fluorochrome is found not only to be a stable marker of the tagged cell, but also to be proportionally, arithmetically diluted into its daughter cells; i.e., if the CFSE-tagged cell divided once, their daughters would contain ½ of the fluorescent signal, their daughters $1/4^{th}$ etc. Thus, if after some time we would still see the cells with the fluorescent signal exceeding ½ of the original, we must assume they did not divide since the tagging operation, so their lifespan has to be at least equal to the period between the tagging and the observation. For murine T-cells it was shown to exceed on average half a year, which for most mice strains is about $1/4^{th}$ of their typical lifespan. This constitutes a proof that at least some murine T-cells may live for a major portion of the animal's life and, possibly, their lifespan would be similar to the lifespan of the mouse.

The same is much more difficult in humans—for obvious reasons we cannot tag and observe our own cells that way. Also, one has to be careful to distinguish between the proliferative lifespan of a T cell—i.e., how long it and its progeny of the same clone would stay in the organism—and the lifespan of a T-cell "as such"—i.e., how long a nondividing T-cell can stay alive and "clog the niche". A dreadful event in recent history actually did the human cell tagging for us. In 1945, citizens of two Japanese cities were exposed to extremely high radiation of atom bombs. Many of those, who survived the holocaust, exhibited various mutations and changes in their genetic material, frequently leading to the development of malignancy (including the leukemias). However, in some of them a special type of chromosomal mutation can be demonstrated; this one leads to the appearance of circular chromosomes and other chromosomal mutation, precluding the symmetrical division of such T-cells [40, 41]. Thus, putting the two together: if the cell with this mutation was generated in 1945 and it can still be detected today, it must be more than 60 years old; in fact,

these are the maximal estimates of the human lymphocyte lifespan based on this singular phenomenon [53–55].

Newer data, utilizing other approaches for establishing the maximal (or average) lifespan of human T-cells are more confounding and yield much smaller values, from a few days or weeks in the case of naïve, to at most a couple of years for the memory T-cells [5, 47, 48] (albeit some papers state that the memory T-cells do not differ or have a shorter lifespan [87]. However, these tests, marking the cells for example with deuterated (^{2}H)-glucose, do NOT actually "see" the marked cells throughout their life, but estimate average lifespans based on incorporation of the ^{2}H-glucose deuterium in the DNA; thus it is rather an average than a maximal lifespan that they estimate. Another popular way of assessing the T-cell clone lifespan is by the estimation of number of population doubling in vitro and then multiplying it by the time required for a single population doubling. This is of course the T-cell proliferative lifespan, not the maximal (or even average) time any single T-lymphocyte may live when undisturbed in an organism. This type of study yields the average lifespan for human memory cells about 15 years and their maximal lifespan of about 35 years [1]. More recent data from the in vitro cultivated T-cell clones show that hey can perform close to 100 population doublings, which would extend their maximal lifespan to that close to observed for T-cells of the A-bomb victims [62, 63]. Concluding, at least some T-cells may stay alive for a long time even if not dividing and thus "clog the niche";—i.e., limit the available space for the progeny of the still-reactive lymphocytes.

As long as the two processes (i.e., production and/or influx of new T-cells and the removal of the T-lymphocytes that are no more needed) are in relative balance, the niche "volume" or total numbers of included T-cells will stay more or less stable (even if the properties (phenotype) of the cells filling it will change, the quantity will be homeostatically maintained). This seems to be true for the fate of T-cell niche in the healthy young individuals (Fig. 3a). Still, all the time one has to be aware that the above description is general (using the keyword "T-cells" rather than alluding to separate subpopulations of these) and that so far there are not much data regarding the behaviour of any single T-cell subpopulation as a niche-filler. One consideration that must be made here and that would impact on the overall reactivity of the T-cell system in the aged (but would not in fact change much the total numbers of T-cells in the niche) would be the slow replacement of many very diversified variants of the T-cell receptor TCR (deciding on the ability of T-cells to react to multitude of previously unknown antigens) by much fewer numbers of these, leading to the phenomenon described as TCR repertoire contraction and resulting in vastly reduced ability of T-cells of even healthy elderly to recognize and react to new antigenic challenges [60].

However, as the organism/individual ages, these homeostats seem to become more and more impaired, as we know from the observational and experimental evidence accumulated so far.

Based on the early studies of the ability of T-cells of old mice and elderly people to proliferate in vitro to mitogenic challenge, it was long ago established that their overall proliferative capacity is significantly dwindling with advancing age. It

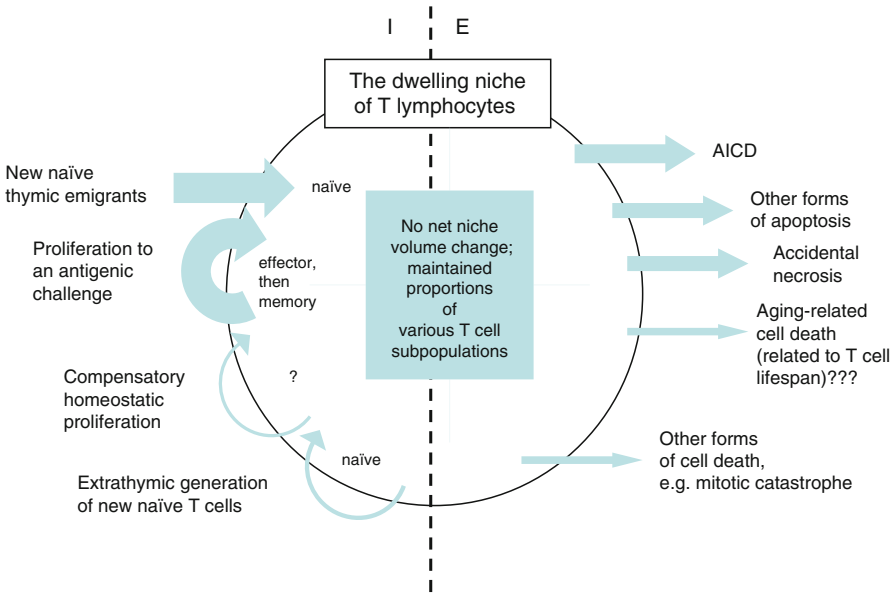

Fig. 3a The T-cell niche homeostasis in young adults: I ≈ E

has been shown many times both for the T-cells of old mice as well as for human peripheral blood lymphocytes (for the review see for example [33, 61]). T-cells of old organisms incorporate less ^{3}H-thymidine when stimulated in vitro with either immobilized anti-CD3/anti-CD28 or with plant mitogens (like phytohaemagglutinin or concanavalin A) or with the "membrane-bypassing" cocktail of calcium ionophore ionomycin and phorbol ester. Also, when their ability to double their numbers in vitro is calculated, it is significantly, much lower than that of young cells. Both these parameters tell us that the general, T-cell population-wide ability to respond to relevant stimuli is decreased in the T-cells of old individuals (reviewed in [20 33]). The net result should be the reduction of the T-cell niche volume/cellularity (Fig. 3b).

One of the problem a researcher of T-cell aging encounters when studying the field is the T-cell phenotypic shift occurring in the elderly. The best known forms of this remodeling are the naïve-to-memory shift and the accumulation of T-cells deprived of CD28 costimulatory molecule [9, 24, 28]. The first, naïve-to-memory shift (or accumulation of phenotypically memory T-cells at the expense of naïve ones) is intuitively obvious: given relatively constant T-cell niche volume and many years of exposure to environmental antigens and pathogens, our adaptive immune system must produce many variants of memory cells left behind after each antigenic challenge, that will take the niche space [16]. In addition, we know for many decades that the main if not sole provider of new naïve T-cells in our youth, the thymus, is grossly reducing its output after the puberty (even if we know now, that it does NOT stop working then; in fact, naïve T-cells and the thymic hormones are

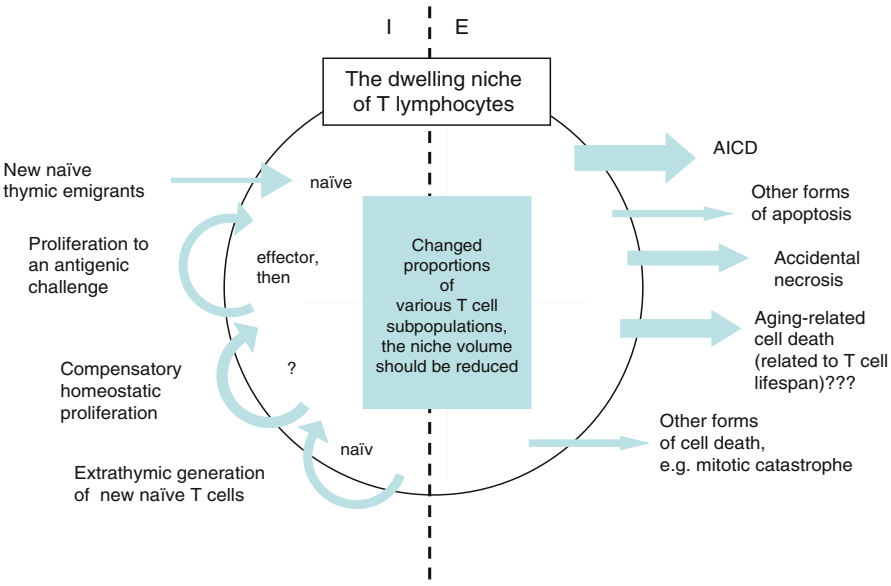

Fig. 3b The T-cell niche homeostasis in elderly: I << E (old paradigm)

produced still when we are approaching old age) [15, 59]. At the very old age the thymus is apparently no more the source of new, naïve T-lymphocytes, yet even in the very old people we can still detect some of them [59, 65]. This leads to the conclusion that either they are the survivors from our youth or middle age (but see the discussion on the T-cell lifespan in this chapter!) or they are maturing from the bone marrow precursors without the need of the thymic microenvironment (extrathymically). The data showing decreased numbers of new thymic emigrants containing the TCR gene rearrangement excision circles or TRECS suggest that in the very old (centenarians) such cells are practically absent [59] which would rather support the first possibility. The question how it in fact is and another—whether these naïve T-cells that appear in the elderly are still fully functional (for instance, can they still divide as dynamically as the naïve T-cells of young individuals when challenged)—remains unanswered so far.

A related factor is that the proportion of T-cells that do not enter division cycle upon stimulation (nonzero even in the young individuals) is vastly increasing among T-cells of old people. These lymphocytes are presumably proliferatively senescent, i.e. post-mitotic and unable to divide anymore. However, it is not known as yet, whether they in fact only "stay there" and "clog the niche", or are they still able to perform some other, nonproliferative functions, like the cytotoxic or regulatory (cytokine- or contact-related) activities. One of the possibilities is that they could be devoid of certain essential molecule (or signalling pathway) necessary for initiation of proliferation; an important candidate here could be the major costimulator molecule of the T-cells, the CD28.

Accumulation of the $CD28^{nul}$ subpopulation in both the $CD4^+$ and $CD8^+$ lymphocyte populations was found to accompany even healthy aging [9, 21, 24, 88, 92]. Certain studies demonstrated that these $CD28^{nul}$ cells have many features ascribed to the aging T-cell population, including the decreased proliferative capability measured by ^{3}H-TdR incorporation [9, 13, 18, 19, 22, 23, 92], contracted T-cell repertoire [78, 89] (see also the chapter by J. Goronzy in this volume) and modified cytokine production [2, 26, 76]. Thus it was assumed that their accumulation might be responsible for the impaired functioning of the T-cell pool in the elderly. However, while $CD28^{nul}$ cells may form even more than 50% of all circulating $CD8^+$ lymphocytes they rarely exceed 10% of the $CD4^+$ cells in a healthy elderly individual [9, 11, 21, 24, 88, 92]. Thus, their accumulation cannot be considered the culprit for grossly decreased proliferation rate of either $CD4^+$ or even $CD8^+$ lymphocytes (even in the latter population, the decrease in 3H-thymidine incorporation is by far more than 50% when we compare cells from young and elderly individuals). Interestingly, even the relatively high accumulation of $CD8^+CD28^{nul}$ lymphocytes in the elderly cannot be responsible for decreased proliferative capacity of the $CD8^+$ population in old people; it was recently found (using the flow cytometric DCT technique utilizing the supravital staining of proliferating cells with the fluorescein derivative, CFSE) that in fact, these $CD8^+CD28^{nul}$ cells do proliferate more and more with advancing age and, when drawn from the blood of oldest old, they can make in vitro many divisions [14]. This was also shown for t he $CD4^+CD28^{nul}$ cells, albeit the latter seem to be less proliferatively active in the elderly than their $CD8^+$ counterparts [12]. Thus, overall effect of accumulation of $CD28^{nul}$ cells on the status of the T-cell pool in the aged (at least for its $CD4^+$ compartment) cannot be that much, unless they would be functional regulators/suppressors. This latter possibility is tempting, however until now (mid-2007) it had not been sufficiently documented, despite showing at least some cytotoxic abilities in them [52, 56, 58].

On the other hand, the existence and potential importance of the $CD28^{nul}$ cells (at least within the $CD4^+$ population of human lymphocytes) may be just the tip of an iceberg, with the most of it metaphorically containing the $CD4^+$ cells with lowered numbers of CD28 molecules on their surfaces, but not lacking them altogether. In fact, we were able to show some transcriptive activity of the CD28 gene by RT-PCR even in the notorious $CD4^+CD28^{nul}$ clones, which suggests that the actual range of CD28 expression level on the human $CD4^+$ lymphocytes might be from near-zero to whatever maximum (Witkowski, unpublished). We have shown before that this is the case: $CD4^+$ lymphocytes of healthy elderly people express on average fewer CD28 molecules per cell than those from healthy young people [12, 93]. This observation is similar to that obtained for the $CD4^+$ lymphocytes of rheumatoid arthritis (RA) patients, considered to show the phenotype of accelerated aging [11]. Decreased numbers of CD28 on elderly $CD4^+$ cells expectedly have their consequence, the CD28 being known as a major costimulatory molecule for these cells. Using advanced DCT technique we were able to show that the CD28 molecules' number inversely correlates with the time, required by the T-cell to exit the resting G_0 phase and to enter their first division upon stimulation [12, 93].

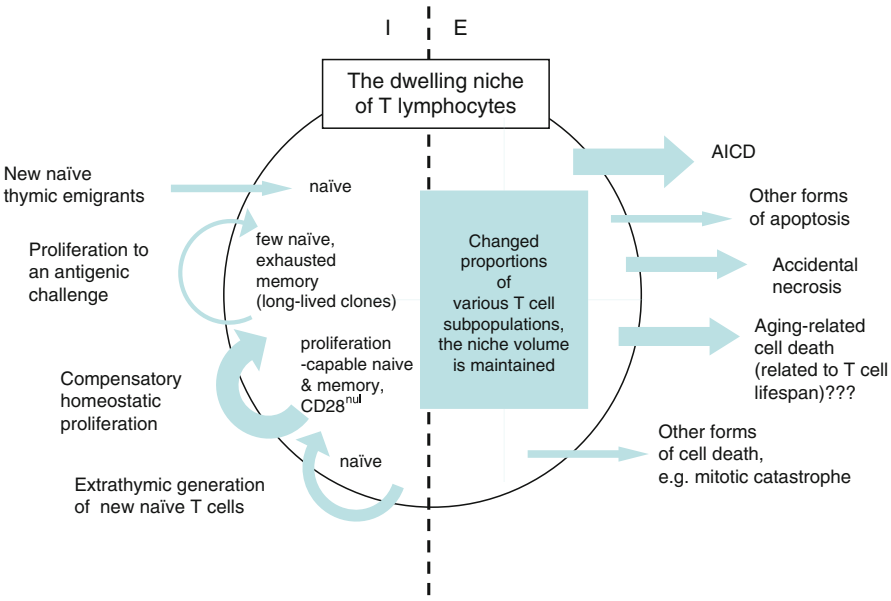

Fig. 3c The T-cell niche homeostasis in elderly: I ≈ E (proposed model)

Summarizing current knowledge, with aging the production of new cells [both naïve (first of all) and effector as well as memory] seems to be reduced, and the accumulation of post-mitotic, senescent T-cells is increased. On the other hand the AICD and, possibly, other forms of apoptosis and—hypothetically—T-cell death related to the more and more of them attaining cellular old age (senescence) and dying of it, as well as possibly the mitotic catastrophe related to accumulating DNA damage are increased. This would result in the net emptying of the T-cell niche, observed as lower numbers of T-lymphocytes in the circulating blood, reduced volume of the T-cell compartments in the lymphatic tissues etc (Fig. 3b). However, these symptoms do not occur, so some mechanism must maintain the filling of the niche (Fig. 3c).

Why do the T-cells proliferate differently in the aged individuals remains not fully understood. It is well established that various facets of the signal transduction mechanisms leading to the turning on of the DNA replication and cell division are impaired in the T-cells of the elderly [31–33] (see also Tamas Fulop's chapter in this book). The major mechanism governing the progression of the cell cycle when it was already initiated consists of the interplay between the cyclin-dependent kinases (the cdks, in the lymphocytes mostly cdk 2, 4 and 6) that are supposed to phosphorylate specific proteins at specific times during the process, the cyclins (A through G, forming regulatory parts of the active phosphorylating complexes, but must be tightly controlled and timely eliminated or the cell may become neoplastic) and the cyclin-kinase complex inhibitors including the p21cip/waf, p16ink4 etc. The latter are already known to accumulate in many cell types of old individuals including

the lymphocytes [36]; ultimately their amount is supposedly that high that the cell cannot perform any cdk-dependent phosphorylation and stops dividing completely, reaching proliferative senescence. On the other hand, the levels of cyclins and the cdks are posing more difficulties for interpretation. It was shown that the levels of cyclins A, G and D (2 and 3) are lowered in the T-cells of old individuals. These observations are related to their increased destruction by the ubiquitin-dependent proteolytic machinery [68–70] and possibly also due to decreased activity of relevant genes [4, 25, 36, 73]. Similarly T-cells of the elderly are containing less cdk kinases, which disrupts the phosphorylation processes needed for cell cycle progression [4, 25, 36, 73]. However, the data available so far do not consider the remodelling of the T-cell pool, including the increase in the proportion of senescent cells. Thus, it is theoretically possible that those T-cells that are still capable to proliferate in the elderly may have these mechanisms even more active than the young ones! In fact this is precisely what we see: using the DCT cytometric technique we had demonstrated more divisions made in vitro by fewer $CD4^+$ cells of the elderly people, associated with increased levels of D1 cyclin (governing the length of the G1 phase of the cycle), both total and cdk-bound in an active complex [93].

This brings us to the whole huge question of possible changes in the cell cycle length and productivity related to aging.

When we consider the actual cell cycle length (or the time between the two consecutive mitoses) we have to bear in mind the serious consequences of shortening or elongation of the cycle by relatively minor span of time per cycle. Using simple enough arithmetic one can show that the cells, for which the cell cycle is shorter, will make more divisions at the same time compared to other with longer cycle. As the average length of the human T-cell cycle is somewhere between 12 and 20 hours [93] and stimulated T-cells are able to perform up to 15 or more division in vitro without artificial support (like feeder cells or IL-2 and other growth factor supplementation), one can easily calculate that after the time the cells with longer cycle spent on x division, those with shorter cycle would make x + 1/cell cycle length * number of cycles until time of observation; eventually, after a precisely calculable time faster cells would make one division more than the slower ones, i.e, at the end of that time, there should be up to twice as many faster cells than slower ones! This may be of physiological importance, for example as a reason for elimination of less active clones by more active ones and changed TCR diversity in the elderly. Thus, it seems important to know if the cell cycle length changes with advancing age.

In fact such studies were performed already a few times for murine and human T-cells from donors of different ages. The methodology applied was mostly staining the cellular DNA after a designed time of stimulation and detecting the proportions of the cells in G0/G1, S, and G2/M stages of the mitotic cycle which, with appropriate analytical tools, allows for approximate estimation of the length of each stage. This technique yielded some intuitively expected results—namely, T-cells from old individuals tended (on average) to have their cell cycle longer than cells from young individuals [4, 25, 73]. While looking at the various stages of the T-cell cycle, various authors reported: no change or elongation of G1 (the latter related to the accumulation of the inhibitor of cyclin D/cdk4/6 kinase complex, the p21 cip/waf (by

BrdUrd/Hoechst staining; [6]) and an elongated S phase (here the example is the Werner's syndrome S phase length [71], but one can easily, intuitively understand that the T-cells (and other cycling cells) of the elderly individuals may have more DNA damage accumulated, and less effective mechanisms of its detection (including the p53 [51]) and repair [3, 39, 77], thus may be prone to slowing down the cycle to allow more time for repairs). Also, and for the same reasons, elongated G2 phase was reported [66, 67]. According to these reports, age-related G2 elongation can be reversed by caffeine which, in turn, is reversed by adenosine (which provokes the—currently unanswered yet—question on the role of changed availability of energy and/or cAMP in the observed process).

Thus, in a model of the T-cell cycle changes related to aging, that arises from the abovementioned observations (Fig. 4a), with aging all or at least some of the cycle phases are elongated, leading to the elongation of the whole cycle. Accordingly, compared to the young, the T-cells of elderly would make fewer divisions in the same time from stimulation and, assuming other factors influencing the progeny number would not change (which is not entirely true, as the level of AICD changes with aging, as do probably other means a T-cell might die) we would see fewer daughter cells at the end of any observation period. This is in agreement with old data on ^{3}H-TdR incorporation in the DNA of mitogen-stimulated T-cells of young and elderly. However, assuming the above as the whole truth we would expect not only the immune response involving the T-cell proliferation to be much less effective (due to not enough effector cells produced on time), but also the numbers of T-cells in the elderly to dwindle quite rapidly, both during activation (AICD and the mitotic catastrophe on the rise) and during the rest period, where the homeostatic mechanisms to fill the niche would also fail. Yet, we do not see a significant change in the volume of lymph nodes, spleen and the MALT when we age, even if their internal histology may change with age [84, 85, 90, 91].

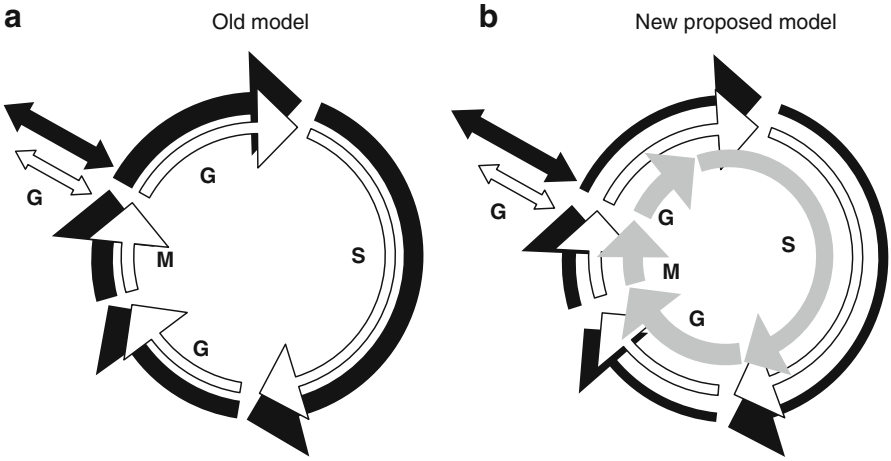

Fig. 4 Changes of the length of cell cycle phases (G0 through M) in the T-cells of healthy elderly. Relative lengths of cell cycle phases of T-cells of young (□), and elderlyindividuals(■—presenescent, ■—homeostatically proliferating). Smaller radius indicates shorter cell cycle

One common mistake in such studies is that the researchers assume some special value for the length of any specific cell cycle stage. For instance, according to Kypreou et al, at 72 hours stimulation (in vitro) the lymphocytes are in the S phase [42]. In fact even if we assume the initial synchronization of these cells' entry in the activation process (by the token of almost immediate contact of all tested cells with the stimulus upon its admixture), we do not know the actual timing of stages preceding the S phase, including first of all the G0→G1 transition as well as the G1 phase itself, which is the most variable and prone to change in length. Thus, any *a priori* assumption of the length of any phase of the cell cycle skews our understanding of possible changes, by not allowing the researcher to assume that any of them might actually be changing with age! As we had demonstrated confirming earlier suggestions, the G0→G1 phase is significantly elongated (in some cases to more than 50 hours!) for the $CD4^+$ cells of healthy elderly and this elongation depends on the availability of CD28 as a source of costimulatory signal [93].

On the other hand, when we applied the abovementioned DCT flow cytometric technique to tag and enumerate dividing human T-cells obtained from people of various age, and the not-so-complicated mathematics for calculations, we have found that, in fact, on average the cell cycle of the $CD4^+$ lymphocytes of the healthy elderly is shorter than that of the same cell type from young people [93]. In detail, the $CD4^+$ population that we studied that way was more diversified: apart from the cells that divided fast (making more divisions per a dividing cell) there were many (more that among the lymphocytes of the young people) those that did not divide at all (senescent?). Also, the shortening of the cell cycle was true for those $CD4^+$ cells that were still expressing some (but FACS-detectable) CD28 on their surface, while the $CD4^+CD28^{nul}$ cells of the elderly divided with the speed not different from the same population dwelling in the young. This observation—in our opinion—is indicating that the cell cycle behavior does NOT undergo any COMMON type of changes in aging, even within such a seemingly uniform class of cells like the T-cells; rather, different subpopulations of the T-cells, including those differing in the expression of CD28, but possibly also the broadly different $CD4^+$ and $CD8^+$, naïve and memory cells etc. would follow their separate patterns of cell cycle change, requiring separate studies. In our opinion this once again describes the $CD28^{nul}$ population as an end product of the process of aging. At the end, of course, all of them would fit into the common pattern of remodeled T-cell niche observed in the healthy elderly.

Thus, we propose another model (which according to our experiments is true at least for the $CD4^+$ lymphocytes) where those T-cells of the healthy elderly that still had not reached proliferative senescence would actually divide faster (their cell cycle would be significantly shorter), while these approaching it, but not yet senescent—slower (Fig. 4b). That way in the same time fewer cells than in the young would make more divisions than the dividing lymphocytes of young individuals and the number of their progeny would remain reasonably similar to that seen in the healthy young people. In our opinion, this model fits well in the aged immune system remodeling theory [27, 30], adding a changing functional component to its—already known—changing phenotypic characteristics. The mixed cell population that fills the T-cell niche in the aged organism would

therefore consist of increased numbers of the progeny of those clones that can still divide relatively vigorously, steadily reduced numbers of those which still divide slowly (presenescent), and relatively constant or slowly rising numbers of senescent (postproliferative) T-cells clogging the niche (resulting *inter alia* in the observed TCR repertoire contraction)—Fig. 3c. Interestingly, homeostatic proliferation—one that plays a role if filling supposedly empty space in the T-cell niche after the demise of exhausted clones—is reported for both the naïve and memory T-cells of the elderly [60].

The model we propose might gain another aspect, related to the concept of inflamm-aging (readiness of the immune system of elderly to initiate the inflammatory reaction or even permanent state of such mild, subclinical inflammation [17, 29, 75]). It was shown earlier that dexamethasone (a synthetic antiinflammatory glucocorticoid) extends the G1 phase of stimulated human lymphocytes and it is suggested that natural glucocorticoids do the same [7, 8]. The levels of glucocorticoids in the sera of elderly people are variably reported as lowered, unchanged or (quite frequently) increased as compared with these observed in young people; the latter are associated with inter alia worsening of the hypothalamic functions (including memory) [49, 50, 81]. However, these data concern mostly the total levels of the hormone and not the levels of its free, active form. Yet, in our opinion, the inflamm-aging state should be associated with lowered levels of **free** glucocorticoids observed in the elderly. Thus, shorter G1 that we suggest as the reason for overall cell cycle shortening of elderly $CD4^+$ cells might be related not only to high D1 cyclin in these cells that we have reported [93], but also to less free glucocorticoids. Our recent work shows very much decreased amount and activity of cellular β-glucuronidase being the product of Klotho gene in the $CD4^+$ lymphocytes of healthy elderly people [94]. Klotho, recently dubbed the aging hormone, is deeply related to the process of aging, mostly due its involvement in the regulation of calcium and phosphate balances [38, 83, 86]. However, the enzymatic activity of Klotho β-glucuronidase is directed *inter alia* towards the steroid glucuronides (a major water soluble conjugate of steroids manufactured in the liver as means of eliminating the hormones with urine and thus regulating their concentration and activity) and thus, when active, it is keeping the free steroid levels up [35]. In the elderly, decreased Klotho expression and activity would not prevent glucuronidation and elimination of glucocorticoids which then exert less antiinflammatory activity (hence inflamm-aging) and less G1 phase elongating activity (hence shorter G1 phase in the 'Klotho-depleted' T-cells of old people). Accordingly, T-cells of old people seem to be less sensitive to antiproliferative activity of cortisol [45]. Interestingly, very recently Klotho has been described as a direct antagonist of the Wnt gene product, at least in certain stem cells [10, 44]. The role of Wnt—related pathways in the development, differentiation and function of human B- and T-lymphocytes is recognized (for the review, see [72, 80]), and its relation to cellular aging on one hand and to the pathogenesis of rheumatoid arthritis on the other at least strongly suggested [37, 57, 79]. Negative association between Klotho and Wnt opens a new, interesting avenue for aging research.

Concluding, despite already broad and constantly increasing knowledge on the aging-related changes in the dynamics of human T-cell proliferation, this knowledge is so far (end 2007) by no means complete and requires much further study.

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Mismatch Repair System and Aging: Microsatellite Instability in Peripheral Blood Cells of the Elderly and in the T-cell Clone Longitudinal Model

Simona Neri and Erminia Mariani

Contents

Abbreviations

ACE	angiotensin converting enzyme
AID	activation-induced cytidine deaminase
APOB	apolipoprotein B
APOC	apolipoprotein C
APOE	apolipoprotein E
CD34	cluster of differentiation 34
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
ExoI	exonuclease I
FES	felin sarcoma oncogene
HLA	human leukocyte antigen
HNPCC	hereditary nonpolyposis colorectal cancer
HRAS	Harvey rat sarcoma oncogene
MLH1	MutL homologue 1
MMR	Mismatch repair
MSH2	MutS homologue 2
MSH3	MutS homologue 3

E. Mariani (✉)
Laboratorio di Immunologia e Genetica
Istituto di Ricerca Codivilla-Putti, IOR
Via di Barbiano 1/10, 40136, Bologna, Italy
Tel.: 0039 051 6366803; Fax: 0039 051 6366807
E-mail: marianie@alma.unibo.it

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MSH6	MutS homologue 6
MSI	microsatellite instability
MtDNA	mitochondrial DNA
p53	polypeptide 53
PBMC	peripheral blood mononuclear cells
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD	population doublings
PMS1	PostMeiotic Segregation 1
PMS2	PostMeiotic Segregation 2
RER	replication error
RFC	replication factor C
RPA	replication protein A
SNPs	single nucleotide polymorphisms
TCC	T-cell clones
TH	thyrosin hydroxylase
TPOX	thyroid peroxidase
VNTR	variable number of tandem repeats
VWA31	von Willebrand A31

Abstract: Age-related accumulation of DNA damage in human T-cells has been well documented and could be associated with T-cell malfunctions. Therefore, an age-related reduction in DNA repair capacity of human lymphocytes may contribute to this phenomenon and play a key role in the modification of the immune response observed in the elderly. Because the Mismatch Repair system is the main post-replicative pathway for the correction of replication errors and few data suggest a possible alteration with age of this repair pathway, it is conceivable that, also in the immune system, age-related alterations of mismatch repair could contribute to the accumulation of genetic damage. This is particularly true for adaptive immune response, whose function depends on the ability of T-cells to undergo repetitive replications after antigenic challenge. The present chapter will focus on the role of the Mismatch Repair System that is recently emerging as a possible additional mechanism contributing to the accumulation of genetic instability during aging in peripheral blood cells. In vivo data at present available in the literature and results from studies on cloned human T lymphocytes cultured for different periods in vitro, as a model of immunosenescence, will be reviewed.

Keywords: Aging • Microsatellite instability • Mismatch repair system • T-cell clones

1 Introduction

The understanding and prevention of age-related diseases rely on the study of the molecular mechanisms underlying the physiological aging process and different theories have been proposed. According to the "soma theory", the aging process is caused by a life-long accumulation of random damages in somatic cells and tissues (Kirkwood, Kowald 1997) compromising the functional activity of cells and ulti-

mately leading to cell death. This indicates a central role for the different mechanisms of cell care and stress response cooperating in the regulation of life span, allowing a definition of the so-called "network theory of aging" that includes the effects of defective mitochondria, aberrant proteins, free radicals and DNA mutations (Kirkwood, Kowald 1997) as contributors to the overall process of senescence.

2 Aging and DNA Damage

DNA damage might contribute to the aging process by interfering with DNA replication and transcription impairing the functional ability of cells and thus leading to a senescent phenotype, loss of cellular function, cell death or tumours (Walter et al. 1997).

A wide range of damages to the native structure of DNA (single and double strand breaks, apurinic and apyrimidinic sites, base alterations, methylation, inter and intra-chromosomal cross links, bulky and smaller adducts and distortion of helix by intercalation) can occur through spontaneous damages arising from byproducts of the cellular metabolism or by exogenous chemical, radioactive, viral and mutagenic agents (Reddy, Vasquez 2005). However, the steady state level of spontaneous DNA lesions is very low and therefore difficult to evaluate, under normal conditions. Experimental results do not show directly that decreased genomic integrity causes senescence of somatic cells, but many studies have demonstrated direct correlations: base adduct levels in nuclear and mitochondrial genomes shorten life span and are related to decreased functions of aging. In addition, chromosome aberrations in human peripheral blood lymphocytes do increase with age (Prieur et al. 1988), as well as mutation frequencies at the level of specific genes (as HPRT locus) (Vijg 2000). In any case, the critical load of cellular mutation able to induce physiological consequences is still undetermined.

3 Aging and DNA Repair

Genetic stability is controlled by a number of cellular functions including DNA replication, repair and recombination complexes. Mutations in DNA repair genes frequently lead to genome destabilization and consequent increases in the frequency of mutations. Since systems regulating genome stability are considered to be major safety systems for longevity, it is likely that the inactivation of one or more of such pathways accelerates both age-related deterioration/death and mutation accumulation, at the same time. Indeed, several studies have addressed relationships between DNA damage, its repair and aging, and have suggested an age-dependent accumulation of DNA damage as partially responsible for the impairment of cellular functions and an increased rate of diseases, such as cancer, in the elderly. The accumulation of DNA damage with age (Walter et al. 1997; Barnett, Barnett 1998; Vijg 2000; Doria, Frasca 2001) seems to affect various tissues at different rates, as observed in trans-

genic mice harbouring the LacZ gene (Ono et al. 2000). In addition, a positive correlation between DNA repair capacity and life-span has been demonstrated (Hart, Setlow 1974). The impact of a malfunctioning DNA repair system on genomic integrity is also evidenced by progeroid syndromes in which mutations in DNA repair genes induce a premature aging phenotype characterised by immune defects and increased susceptibility to cancer development (Bohr 2002).

3.1 DNA Repair Pathways

Mammalian DNA repair processes depend on a number of complex pathways to cope with lesions in DNA structure. At least four main pathways have been described so far:

a) the Direct Reversal Repair pathway catalyses a direct reversal only involving single enzymes (e.g., alkyltransferase, removing the methyl group from O6-methylguanine) and DNA ligase (rejoining single-strand breaks) (Harris et al. 1983);
b) the Excision Repair pathway is the predominant mechanism for the maintenance of genomic integrity. This pathway repairs different DNA lesions, ranging from simple base methylations to interstrand adduct formation resulting in major distortion of the DNA structure. Two distinct systems belong to it:

– Nucleotide Excision Repair, which corrects a broad spectrum of structurally unrelated lesions such as UV-induced photoproducts, chemical adducts, intra strand crosslinks and some form of oxidative damage. It can repair any part of the genome, however, damage recognition and repair of trascriptionally active genes (Wood et al. 2001) is performed preferentially by an alternative pathway, termed transcription-coupled repair (Hanawalt 1994);
– Base Excision Repair, which is, perhaps, the most fundamental and ubiquitous DNA repair mechanism in all higher organisms that depend on oxygen for living (Wilson, Bohr 2007). It has evolved to handle the numerous minor alterations (such as spontaneous modification, oxidation, deamination and loss of bases) that can occur in the structure of DNA as a result of cell metabolic activity. This kind of repair is important in post-mitotic tissues, where simple base modifications are likely more prone to occur than major damages;

c) the Recombination Repair pathway that corrects DNA double strand breaks frequently arising from the stalling of the replication fork and from the attack of exogenous agents (such as ionising radiation or chemicals), inducing interstrand or intra-strand cross links and preventing the use of one of the strands as a template for the repair process (Thompson, Schild 2002). Two types of Recombination Repair are described:

– Homologous Recombination, a complex and poorly understood process that entails an intact homologous DNA strand as a template to repair DSB (Sonoda et al. 2006);

– Nonhomologous End Joining that, by contrast, entails relegation of the broken ends without respecting homology and is consequently relatively error prone. Nevertheless, it is a major pathway for double strand break repair in mammalian cells and is thought to be of vital importance in post mitotic tissues (Sonoda et al. 2006).

d) the Mismatch Repair pathway that corrects mispaired bases occurring most frequently during replication (Kolodner, Marsischky 1999).

Finally, the discovery of a number of novel DNA polymerases with the ability to carry out DNA synthesis across a damaged or altered base added new possibilities for understanding DNA repair mechanisms in mammalian cells. These polymerases have different substrate specificities, enabling them to deal with many different types of damaged bases, a process known as translesional synthesis (Rattray, Strathern 2003; Lehmann 2006).

3.2 The Mismatch Repair Pathway (MMR)

The Mismatch Repair system is the main post-replicative pathway for the repair of mismatched DNA (base-base mismatches and insertion/deletion loops occurring during replication, homologous recombination and DNA damage) (Kolodner, Marsischky 1999) and it is essential for maintaining the stability of the genome during repeated duplications. Essential components of the MMR system were identified in *Escherichia coli* and their main activities are reported in the Table 1.

All eukaryotic organisms have MutS (MSH2, MSH3 and MSH6 genes) and MutL (MLH1, PMS1 and PMS2—PostMeiotic Segregation 1 and 2 genes) homologues (Wood et al. 2001; Modrich, Lahue 1996), acting in form of heterodimers, in contrast to bacteria in which MutS and MutL function as homodimers.

In humans, DNA mismatch repair confers to the genome a 100–1,000 fold protection against replication-induced mutations (Loeb 1994). The initial recognition of mismatches is carried out by MutSα and MutSβ, functional heterodimers of Msh2 bound to either Msh6 or Msh3, respectively. They display some functional overlap, with MutSα playing the major role in mismatch correction and being prevalently expressed in the cell. In the following step, MutLα or MutLβ (heterodimers of Mlh1

Table 1 Principal components and functions of the bacterial Mismatch repair system

MutS	Detects mismatches in DNA duplex and initiate the MMR machinery
MutL	Makes a connection between the recognition of a mismatch and its excision from the strand within which it is contained
MutH	Cleaves hemimethylated GATC sites for excision of mismatch-containing strand and formation of nick
Uvr/Helicase	Enters into the nick generated by MutH together with single-stranded DNA-binding proteins

bound to either Pms2 or Pms1, respectively) mediate the recruitment of additional proteins for the completion of the repair process, giving rise to the excision of the mutated strand in either direction to the mismatch and to the resynthesis of the correct sequence (Kolodner, Marsischky 1999). Efficient DNA mismatch repair requires the combined functions of MutS and MutL. The other proteins involved in the repair are: PCNA (Proliferating Cell Nuclear Antigen), whose activity increases the binding of MutSα to mismatched DNA suggesting a role of this protein in the recognition stage (Flores-Rozas et al. 2000; Lau, Kolodner 2003); ExoI (exonuclease I); RPA (replication protein A) and RFC (replication factor C). Once the mutated strand is excised beyond the mismatch, polymerase δ resinthesizes DNA and the nick is sealed by DNA ligases not yet identified (Jun et al. 2006) (Fig. 1).

In addition to a role during replication, MMR proteins have been reported to have other important functions, such as: antirecombination activity between divergent

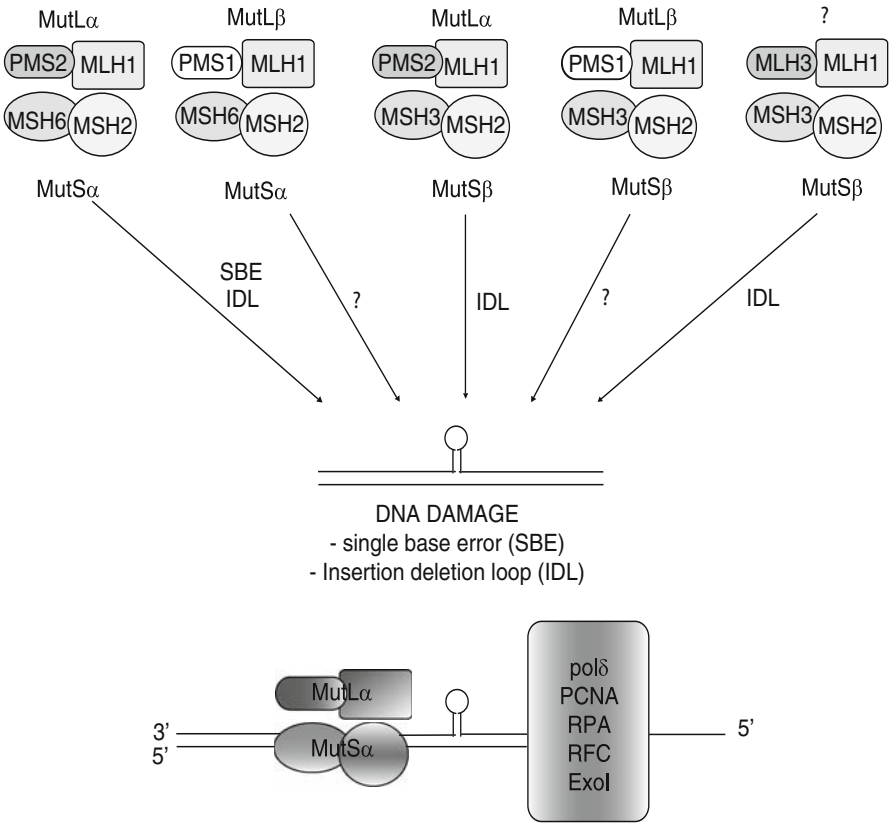

Fig. 1 Schematic representation of the Human DNA Mismatch Repair System. MutS heterodimers (MSH2-MSH6 or MSH2-MSH3) combined with heterodimers of MLH1 with PMS2, PMS1 or MLH3 have different specificities for DNA mismatches or loops (upper panel). Correction is targeted to the primer strand possibly through the interaction with PCNA and additional factors are required to complete the process (lower panel). polδ = polymerase δ; PCNA = proliferating cell nuclear antigen; RPA = replication protein A; RFC = replication factor C; Exo I = exonuclease I

sequences, promotion of meiotic crossover, DNA damage surveillance and diversification of imunoglobulins (Jun et al. 2006). The involvement of MMR in DNA damage response is evidenced by the fact that MMR-defective cells are resistant to alkylating and other DNA damaging agents (Fink et al. 1998). In fact, DNA damage triggers MMR-dependent G2/M arrest, followed by the induction of MMR-dependent apoptosis p53- or p73-mediated. The hypothesis for how MMR is involved in somatic hypermutation and class switch recombination is that, after generation of mutations by AID (activation-induced cytidine deaminase), MMR proteins are recruited to the mismatched DNA and resynthesise the DNA strand with the help of an error-prone polymerase such as polymerase η (Wilson et al. 2005).

Defects in MMR correction pathways are associated with a substantial destabilization of microsatellites, highly polymorphic, tandemly repeated sequences (from one to six bp) interspersed in the genome and particularly prone to slippage during replication. Slippages determine changes of allele length either for insertion or deletion of repeated units. The experimental evidence of this phenomenon is called microsatellite instability (MSI), that is the appearance of additional bands of different lengths or modification of the expected ones. Mutations are observed in repeated sequences, but can also occur randomly in all the genome; therefore, MSI indicates a higher susceptibility to mutations.

Mutator phenotypes due to inactivation of MMR were initially described in HNPCC (hereditary nonpolyposis colorectal cancer) caused by germ line mutations in several members of MMR genes (MLH1 and MSH2 in about 90% of cases), inducing accelerated mutations in microsatellite sequences compared to normal DNA, the so-called replication error (RER) phenotype (Aaltonen et al. 1993; De la Chapelle 1995). Subsequently, it was also described in cancer cell lines and in sporadic cancers of the colon, cervix, endometrium, pancreas, lung, prostate and stomach (Eshleman et al. 1995; Modrich 1996; Kane et al. 1997), due to somatic mutations in MMR genes or, more frequently, to epigenetic mutations, in particular hypermethylation-mediated gene inactivation (Liu et al. 1995; Liu et al. 1996; Moslein et al. 1996; Kane et al. 1997; Herman et al. 1998; Kolodner, Marsischky 1999; Suzuki et al. 1999).

4 Genetic Damage and Immune System

The immune system develops an enormous number of genetically different cells generated by breaking and rejoining DNA sequences coding for antigen receptors, by adapting the DNA repair mechanisms normally used to maintain genome stability. Small populations of naïve and memory T-cells, in order to ensure a correct immune response, have to expand clonally upon antigen stimulation. The ability to expand may depend on the amount of accumulated genetic damage and processes limiting T-cell proliferative capacity might impair the overall immune response. The overlap between DNA repair and immune system efficiency is evidenced by the fact that individuals with defective DNA repair pathways frequently show immunodeficiency.

In addition, immune system efficiency is affected by aging, particularly the T-cell compartment. The impact of age-related immune alterations on lifespan and diseases is in accordance with results from studies in centenarians showing that healthy individuals who have reached the extreme limit of human life in good clinical conditions are equipped with well preserved and efficient immune defence mechanisms (Franceschi et al. 1995).

An age-related accumulation of DNA damage and mutations in human T-cells has been well documented and could be associated with T-cell dysfunctions; it follows that a reduction in DNA repair capacity of human lymphocytes may contribute to this accumulation of DNA damage with age and may play a prominent role in the deterioration of the immune response observed in the elderly and to the development of age-associated immune malfunctions possibly affecting lifespan.

5 MMR System and Aging

MMR deficiency inducing high levels of mutations may only increase the rate of cancer, but not aging. Since cancer is one of the most important causes of mortality in the elderly, it is possible that alterations of the MMR system occurring with age predispose to cancer. Indeed, some emerging evidence indicates that MMR efficiency might be impaired in normal somatic cells with progressive aging.

Msh-2 deficient mice die within one year of cancer with lymphomas (also a common cause of death in aged mice) (Reitmar et al. 1996), while in the first year of life no difference was observed between wild type and Msh2 heterozygotes. MSH2- and PMS2-deficient mice crossbred with transgenic mutation reporter mice generate animals with elevated spontaneous point mutation frequencies in several organs and tissues (Andrew et al. 1997; Narayanan et al. 1997). However, at present nothing is known about mutations accumulated at later ages, since complete lifespan studies on these mice have not yet been performed.

Toyota et al. (1999) found an age-related methylation of CpG islands in normal colon cells affecting different DNA promoter regions, including MLH1 promoter. A large number of CpG in the human genome are progressively methylated during the aging process and, for many genes, this methylation process correlates with reduced expression. The phenomenon appears to be physiologically induced because it is very frequent, it affects large numbers of cells, and it is present in colon tissue from healthy donors and in residual normal colon tissue from cancer patients. The age-related methylation of MLH1 promoter in cancer cells suggests therefore a decreased activity of the MMR system predisposing the elderly to malignant transformation in the colon. In agreement, a spread of methylation in the MLH1 promoter in the normal colonic mucosa closely associated with age and with the development of sporadic MSI in colorectal cancers was found (Nakagawa et al. 2001). The hypermethylation of the MLH1 gene promoter occurring with age correlates with inhibition of its expression and the appearance of MSI.

The frequency of MSI in the pathologic tissue of patients suffering from gastric lymphoma showed a tendency to increase with age, as did microsatellite variability (Starostik et al. 2000).

5.1 Analysis on Peripheral Blood Cells

The immune system, whose impairment is documented with age, is also a possible target of the MMR deficiency, in particular the adaptive immune response that depends on the ability of T-cells to undergo consecutive replications after antigenic challenge. This prominent proliferative stress presumably renders T-cells more prone to possible inefficiencies of DNA repair systems and replicative senescence. In recent years, some data are emerging on age-dependent alterations of the MMR pathway in peripheral blood cells.

A preliminary study demonstrated an age-associated MSI by analysing eight different microsatellite loci on DNA from peripheral blood cell samples from young and old healthy subjects obtained at a ten-year interval (Ben Yehuda et al. 2000). A significantly higher rate of MSI after ten years (in 40% of the loci tested and in 45% of the subjects) was found in older individuals, whereas no difference between paired samples of any of the young subjects was observed. An overall genomic instability in the elderly was subsequently confirmed with an additional panel of microsatellites, together with the lack of an association between MSI and methylation of MLH1 or MSH2 promoters (Krichevsky et al. 2004).

The possible involvement of the MMR system in the accumulation of genetic damage with age was also studied in peripheral blood cell DNA from a wide survey of differently aged subjects (Neri et al. 2005). Five polymorphic microsatellite loci (CD4, p53, VWA31, TPOX and FES), in accordance with the international criteria for the study of MSI in cancer (Boland et al. 1998), were analyzed to find possible age-related instabilities or modifications in allele frequencies. Indications of instability were supplied by both altered allele frequencies in different groups of age and the appearance of trizygosis (three alleles at one locus instead of one or two). Excluding the appearance of plurizygosity, that represents a direct indication of instability, but whose frequency is expected to be low in healthy subjects without germline mutations in MMR genes, in this study it was not possible to compare the allelic pattern with a control (as between normal and tumour DNA from the same patient). For this reason, shifts in allele length, evaluated in terms of age-related modifications of allele frequencies, gave only an indirect indication of genetic instability, possibly due to defective mechanisms of genomic conservation, such as MMR pathway, with progressive aging.

The VWA31 microsatellite showed a significant shortening with increasing age. VWA31 and FES microsatellite alleles presented peculiar distributions in differently aged groups, further suggesting modifications in microsatellite stability as shown by shifts in patterns of allelic frequencies from young (considered as basal condition) to old populations. Only the FES locus, (the most unstable among the

five analyzed), resulted trizygotic in five samples among the more than two hundred analyzed. All samples that were trizygotic belonged to the old and the centenarian groups, while no young subjects ever showed this pattern (Fig. 2). In addition, the majority of trizygotic centenarians displayed, among the three, a rare allele, never observed in homo- or heterozygosis (Neri et al. 2005).

These data show both an increased instability in very advanced age and an age-dependent genetic damage affecting repeated sequences (whose stability is predominantly guaranteed by the MMR system), or a weaker ability to balance the increased rate of genetic damage due to advancing age. Cells repeatedly undergoing proliferation were more exposed to the repair activity of the MMR pathway and possibly, due to its inefficiency, accumulated a greater genetic damage than naive cells. A basic characteristic of immunosenescence is the decline of naive T-cells as well as the accumulation of specific T-lymphocyte clones, mostly of memory and effector T-cells, due to persistent exposure to different antigenic challenges (Franceschi et al. 2000; Globerson, Effros 2000) (Epstein-Barr virus and cytomegalovirus, being the most frequent) (Wedderbrun et al. 2001; Ouyang et al. 2004). It is conceivable that these clonal cells, repeatedly expanded in vivo, may have progressively incorporated a genetic damage not evident in young subjects that, conversely, present a prevalent naïve phenotype. The presence of such populations, frequently dramatically expanded, (Ouyang et al. 2004) could justify the finding of additional allele bands (trizygosis) in DNA from heterogeneous populations of circulating peripheral blood cells. In fact, to be detectable, mutated alleles should be present in an adequate amount of cells, since the appearance of new alleles might be undetectable in poorly represented cells (less than 5–10%) among a mixed population, because of the overloading amount of normal alleles, therefore inducing an underestimation of MSI.

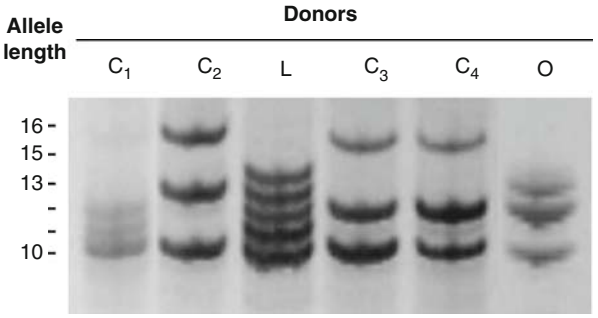

Fig. 2 Trizygosity observed at the FES locus in DNA from peripheral blood lymphocytes of four centenarian (C1-C4) and one old (O) donors. The presence of three alleles at one locus indicates heterogeneity among the analyzed cells, presumably acquired during in vitro replication and giving a different genotype to a portion of cells. Total DNA was amplified by PCR with primers specific for FES microsatellite sequence, then products were electrophoresed on polyacrylamide gel and silver stained. Allele length is indicated on the left and refers to the number of repeats. L= allelic ladder. (from Neri et al. 2005)

As far as the higher instability at the FES locus is concerned, a different sensitivity of this region to the assumed inefficiency of the MMR system is suggested by the evidence that not all sequences are susceptible to, or show the same rate, of MSI. In agreement, a specific FES somatic instability was described in sporadic gastric cancer (Silva et al. 1997) and in lymphocyte clones after in vitro aging (Neri et al. 2004). It is also possible that some alleles undergoing an age-related selection, tend to disappear in the most advanced ages due to a relationship with the FES microsatellite sequence, or with other sequences in linkage disequilibrium with the FES one. The analyzed FES microsatellite lies at intron five of the coding region of FES proto-oncogene that encodes for a nonreceptor protein-tyrosine-kinase whose activation can mediate cellular transformation. Several growth factors, cytokines, immunoglobulin/receptor pairs trigger the activation of cellular FES, shown to play important roles in the regulation of inflammation and immune response (Greer 2002; Yates, Gasson 1996), particularly for survival and terminal differentiation of hematopoietic myeloid lineage (Manfredini et al. 1997). Taking into account the involvement of this factor in the homeostasis of the immune system and modifications of FES-associated microsatellite allele distributions with age, a possible relationship between allelic variants and aging cannot be excluded. Since FES microsatellite alleles are nonexpressed, modifications in allele frequencies might depend on coding sequences in linkage disequilibrium with the analyzed ones, but also on the correlation between the number of repeats and the aging process, thus influencing for example the expression of the gene they are located on, as proposed for VNTR sequences (Bennet et al. 1995). Accordingly, polymorphic alleles of inflammatory cytokines play an important role in age-related chronic inflammatory response diseases, by determining changes in cytokine production (Lio et al. 2002). In addition, it cannot be excluded that polymorphic variations at multiple loci might have produced genotype characteristics contributing to longevity; indeed, a strong familial component of longevity was observed in centenarians (Perls et al. 1998). Different frequencies of variant alleles would indicate a potential functional advantage of those alleles that are more frequent in the disease-free long-lived individuals, as suggested by associations between longevity and allelic variants for polymorphic markers at specific loci such as HLA (Takata et al. 1987), ACE (Schachter et al. 1994), APOB (Kervinen et al. 1994), APOC (Louhija et al. 1994), APOE (Kervinen et al. 1994; Louhija et al. 1994; Schachter et al. 1994), TH and APOB-VNTR (De Benedictis et al. 1998), HRAS1-3'VNTR (Bonafé et al. 2002), as well as mtDNA haplogroups (De Benedictis et al. 1999). Recently, an association between MLH1 gene and longevity was found in centenarians (Kim et al. 2006). In particular, polymorphisms of MLH1 seemed to influence genomic stability and thereby lifespan. By analyzing three SNPs (single nucleotide polymorphisms) leading to amino acid substitutions, a significantly more represented haplotype was found in centenarians than in controls. On the contrary, CD4 (Neri et al. 2005) and p53 (Bonafè et al. 2002; Neri et al. 2005) microsatellites appeared to be stable in different studies, allowing the exclusion of a role of variants of these genes in age-related mortality to such an extent as to alter gene frequency in old people and centenarians.

5.2 The T-cell Clonal Model

To overcome the possible bias of underestimating instability in poorly represented cells, because of the overloading amount of normal alleles, Parsons et al. (1995) performed the analysis on highly diluted peripheral blood lymphocytes in order to amplify the DNA corresponding to maximum three genome equivalents. By analogy, Coolbaugh-Murphy et al. (2005) developed "small pool" PCR for sensitive and quantitative analysis of MSI in somatic tissues by diluting DNA and subsequently amplifying by PCR so that each small pool contained less than a single genome equivalent. Rare mutant fragments contained in one or more small pools would not be overwhelmed by progenitor fragments and could be readily amplified and identified. By using this technique, significant differences in MSI frequencies in DNA from differently aged groups and a positive correlation between age and MSI phenotype were found (Coolbaugh-Murphy et al. 2005). In addition, the frequency of mutant fragments linearly increased with age in peripheral blood lymphocytes from normal individuals, indicating an age-dependent alteration of MMR efficiency.

Long-term CD8+ cell cultures from aged donors undergoing repeated duplications develop MSI as in vitro cell senescence progress, while no MSI develops in young-derived CD8+ T-cells (Krichevsky et al. 2004).

Other studies overcame the problem of heterogeneous cell population analysis by using CD4+ T-cell clones (TCC) (Krickevsky et al. 2004; Neri et al. 2004; Neri et al. 2007). This model allows the longitudinal follow-up of a homogeneous cell population and the functional analysis of a single cell type that, spending its finite lifespan in vitro, provides both important knowledge related to T-cell immunosenescence in vivo and a system to study ways of modulating the aging process (Pawelec et al. 1998; Pawelec et al. 2002). The advantage offered by the analysis of a homogeneous cell population, allowing to join microsatellite instability to increasing duplications in culture, increases the likelihood of detecting new alleles in usually underrepresented cell populations. Indeed, consecutive antigenic stimulation in vitro imposes a marked replicational stress on T-cells, mirroring the antigenic challenge in vivo, as well as, the culture over the entire clonal replicative lifespan mimics the chronic stress thought to really contribute to the possible clonal exhaustion (Hadrup et al. 2006).

Available data indicate that MSI develop with increasing in vitro culture senescence in CD4+ T-cell clones (Krickevsky et al. 2004; Neri et al. 2004), involving different microsatellite sequences, suggesting that a progressive impairment of the MMR system may contribute to the acquisition of genetic damage during chronic antigenic stress in vitro, a phenomenon thought to be of great importance for immune response physiology (Pawelec et al. 2005; Hadrup et al. 2006).

In addition, by a modification of the alkaline comet assay, a reduced ability to repair acridine ICR-191-induced DNA mismatches was observed with aging in culture, indicating that MMR capacity may become deficient in clonal T-cells when they are challenged with supra-physiological levels of DNA damage (Annet et al. 2005).

Moreover, no MSI was observed at increasing population doublings in TCC from young donors, even almost at the end of their finite lifespan in culture (Neri et al. 2004), suggesting an additional relationship between MSI and donor age, in agreement with in vivo data (Ben Yehuda et al. 2000; Krickevsky et al. 2004; Coolbaugh-Murphy et al. 2005; Neri et al. 2005).

Furthermore, microsatellite instability was particularly evident in clones obtained by CD34+ progenitor cells, after undergoing repeated duplication in culture, indicating also the influence of the cell type in addition to in vitro proliferation and aging (Fig. 3).

This may suggest that the efficiency of the MMR system is already optimal in mature T-cells, but that it is less efficient in CD34+ progenitors due to their maturation stage and requirement for differentiation to T-cells in vitro and not in the normal in vivo environment (Pawelec et al. 1998); indeed, early progenitors are

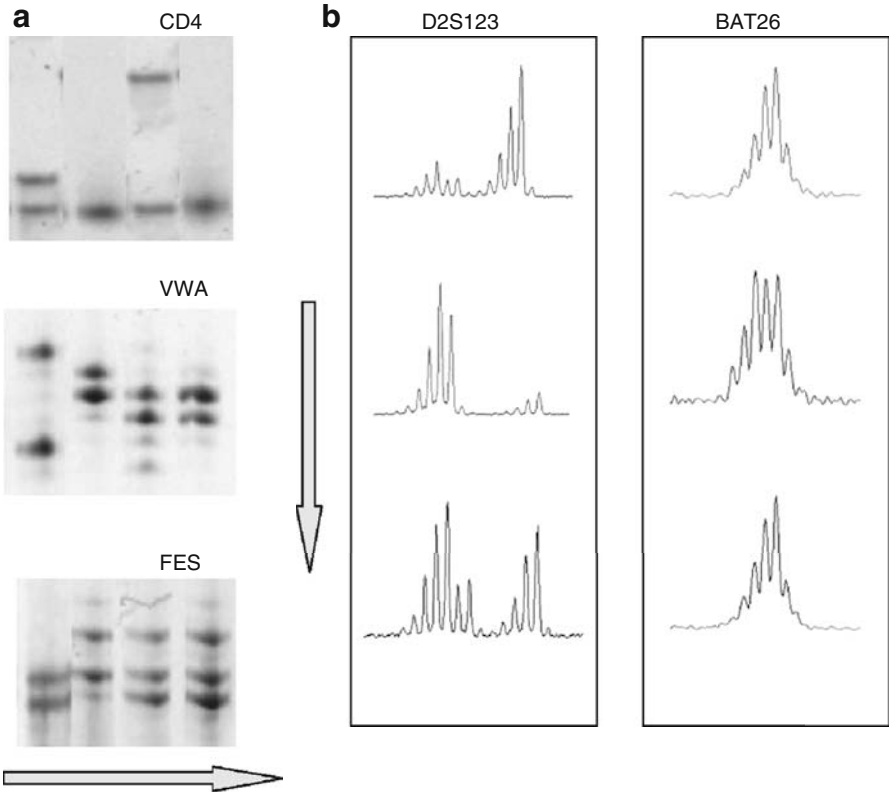

Fig. 3 Example of MSI evidenced during in vitro culture of one clone obtained from CD34+ precursors. CD4, VWA, FES, D2S123 and BAT26 allelic patterns, determined after different population doublings (PD) in culture, are shown. Genotyping was done: a: by PCR followed by analysis on standard acrilamyde gels for CD4, VWA and FES microsatellites; b: by analysis on an automated DNA sequencer for D2S123 and BAT26 microsatellites. Modifications of band length or peak position appear at increasing PD (grey arrows) (Neri et al. 2007)

highly proliferative and, during this period, most susceptible to DNA damage (Park, Gerson 2005). Maturation-dependent alterations in DNA repair function have been demonstrated for the lymphohematopoietic system in association with shifts in DNA repair gene expression profiles (Bracker et al. 2006). In addition, CD34+ cells might have lower levels of genetic integrity control, because they perform T-cell receptor rearrangement in culture. On the other hand, a role of the MMR system in general recombinational processes, VDJ hypermutation and class-switch recombination is well documented (Cascalho et al. 1998; Bellacosa 2001; Larson et al. 2005). Finally, it cannot be excluded that the in vitro system is unable to remove cells that acquired genetic alterations as in vivo occurs by apoptosis. The high instability found during in vitro culture of CD34+ cell-derived clones may suggest the need for particular care for the clinical use of these cells, such as in stem cell transplantation.

The observed MSI did not depend on modifications in the methylation status of MLH1 and MSH2 promoters, as assessed by methylation-specific PCR following bisulfite treatment of clone DNA and no association among methylation status, MMR gene expression, advanced population doublings or presence of MSI was found (Krichevski et al. 2004; Neri et al. 2007).

Semi-quantitative real time RT-PCR showed that transcript levels of the six MMR genes were similar to those observed in total RNA from normal PBMC. MSH6 RNA showed a progressive increase until about 50–60 PD, followed by a slow decrease, while MSH3 mRNA exponentially increased until the more advanced PD, thus suggesting a possible shift from MutSα to MutSβ heterodimer at advanced culture passages. MLH1 RNA did not change significantly during PD, while PMS2 and PMS1 RNA levels increased exponentially (Neri et al. 2007). During aging in culture, unstable clones often presented unstable or decreasing levels of expression, while stable ones constantly increased their expression levels, consistent with a relationship between MMR gene expression, PD and MSI (Neri et al. 2007). In agreement, multivariate regression analysis identified advanced PD as one of the predicting variables for FES MSI, trizygosis and intra-donor changes among clones. MSI and MMR gene expression at the mRNA level were found to correlate, mostly due to a reduced expression of the components of MutL heterodimers, pointing to a role of MMR in the acquisition of DNA damage with in vitro aging: the MutS components MSH6 and MSH3 appeared to be slightly increased in unstable clones; in contrast, the MutL components PMS2 and PMS1 showed decreased transcript levels in unstable compared to stable clones (Neri et al. 2007). This might suggest that unstable TCC maintain the ability to interact with mismatched DNA via MutS, but have a reduced capacity to recruit the enzymes necessary to complete the repair via MutL complexes. Therefore, the upregulation of MutS components in unstable clones, possibly in order to correct DNA mismatches occurring during in vitro proliferation, seems to be not accompanied by an upregulation of MutL components leading to MSI for a reduced expression of PMS2 and PMS1. However, additional posttranscriptional regulation of these genes cannot be definitely excluded. Only limited information is available thus far on the genetic regulation of the MMR system. In MMR proficient cell lines, the regulation seems primarily at the transcriptional level, but mutational inactivation of the components of the system leads

to posttranslational down-regulation of heterodimerizing partners. MMR activity appears to be strictly regulated and modulated by changes in gene expression as demonstrated by MSI induction for loss of MLH1 expression secondary to promoter-hypermethylation (Herman et al. 1998) or by overexpression of MLH1 and MSH3 genes (Marra et al. 1998; Shcherbakova, Kunkel 1999). Therefore, it cannot be excluded that the upregulation of RNA for the MutS components is induced by a defect in the corresponding proteins.

Concerning a possible effect of donor age on MSI, clones from centenarians (an example of successful aging and of preserved immune function) presented a level of instability very similar to young-derived clones, despite the presence of significantly higher levels of MMR transcripts. In contrast, clones from old subjects presented a higher instability compared to young ones, but similar levels of MMR gene expression (Fig. 4). This might reflect the importance of up regulating MMR genes in order to maintain genomic stability and to correct DNA errors accumulating with age, thus suggesting a protective effect of higher MMR transcript levels on genomic integrity.

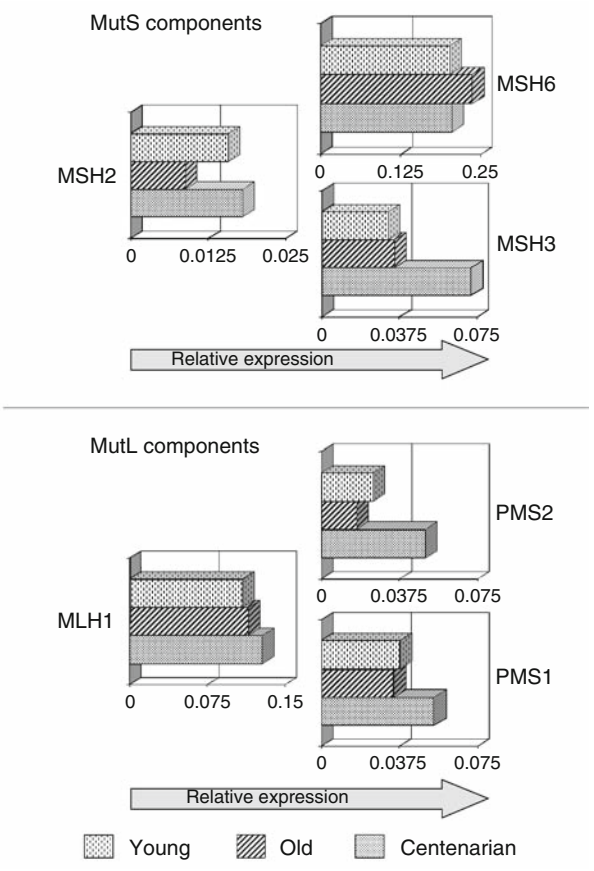

Fig. 4 MMR gene expression at mRNA level (medians) during culture aging of TCC from young, old and centenarian donors (from Neri et al. 2007)

The evidence of differences in allelic patterns among different clones from the same donor suggests the acquisition of new alleles in previous not analyzed culture passages or even before cloning, further supporting the accumulation of MSI even in vivo, as suggested by studies on peripheral blood cells (Ben-Yehuda et al. 2000; Coolbaugh-Murphy et al. 2005; Neri et al. 2005).

In conclusion, it appears that in vitro aging leads to an accumulation of genetic instability manifesting as MSI, possibly to a different extent, depending on cell type, and/or that repeated replication could lead to an accumulation of genetic alterations not counteracted by the MMR system. The correlation between MMR gene expression levels and MSI appeared mostly due to a reduced expression of the components of MutL heterodimers. However, the involvement of other repair pathways (Guo, Loeb 2003) or a possible alteration in polymerase functional activity (Srivastava, Busbee 2003) cannot be excluded. Senescence-associated MMR alterations might also be induced by defects of nuclear localization, assembly and activity of the proteins of this pathway, therefore studies on MMR gene protein levels and functional activity of the MMR system could help in the understanding of these alterations associated to senescence, likely critical for appropriate adaptive immune response.

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Activation-Induced Cell Death of T-cells in Elderly

Ewa Sikora and Agnieszka Brzezińska

Contents

Abstract: The elimination of expanded T-cells at the end of immune response is crucial to maintain homeostasis and avoid any uncontrolled inflammation. Resting mature T-lymphocytes when activated *via* their antigen-specific receptor (TCR) and CD28 coreceptor start to proliferate and acquire resistance to apoptosis. Reactivation of T-cells induces expression of CD95L which after binding to CD95 surface-expressed death receptor triggers signaling pathway to apoptosis. The process is named Activation-Induced Cell Death-AICD. However, in executing AICD death receptor-dependent apoptotic pathway (extrinsic) can overlap with mitochondrial (intrinsic) signaling to apoptosis. Immunosenescence leads to the shrinkage of T-cell repertoire due to the reduction of naïve cells and accumulation of oligoclonal CD8+ and to a lower extent CD4+ cells, which are mainly CD95-positive and CD28-negative. Also, propensity to undergo apoptosis changes with age. However, data so far collected are inconclusive as they show an increased, unchanged or decreased propensity to AICD in the elderly in comparison with young individuals.

1 Introduction

Precursor T-cells from the bone marrow enter the thymus, where they undergo negative or positive selection to produce CD4+ and CD8+ mature cells with diverse functions in the peripheral immune system [34]. In the periphery, T-cells are resting until they encounter foreign antigens and gain the ability to proliferate, differentiate into effector cells, produce cytokines and eliminate target cells. T-cell activation is induced by signal receive through the TCR (T-Cell Receptor) activated by the antigen

E. Sikora (✉) · A. Brzezińska
Molecular Bases of Aging Laboratory
Nencki Institute of Experimental Biology, Polish Academy of Sciences
Pasteura 3, Warsaw Poland
Tel:+4822 5892436
Fax: +4822 8225342
E-mail: e.sikora@nencki.gov.pl

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presented by APC (Antigen Presenting Cells) in MHC context, and costimulatory molecules, including CD28, adhesion molecules and cytokines (IL-2, IL-13, IL-15). This clonal expansion phase is followed by the contraction phase in which T-cell numbers decline to maintain homeostasis and avoid any uncontrolled inflammation. The majority of activated T-cells die by apoptosis and only a few T-cells that have been exposed to the antigen remain. These cells develop into apoptosis-resistant memory T-cells. The mechanism of memory T-cells' survival is not fully recognized, but it can be controlled by cytokines [27, 46]. Thus, T-cell activation is highly regulated and requires a switch from an apoptosis-resistant (clonal expansion phase) towards an apoptosis-sensitive state (elimination phase). The process in which expanded cells are eventually eliminated is named Activation-Induced Cell Death (AICD).

The term AICD was proposed by Green's group, when they showed that T-cell hybridomas or thymocytes died by apoptosis following activation through their CD3 molecules [44]. It is now know that AICD of hybridomas and of activated T-cells is driven by so called death receptors, such as CD95 (another name is Fas receptor) or the tumor-necrosis factor receptor (TNFR) which, once engaged, activate downstream pathways that lead to cell death by apoptosis [26].

An alternative pathway to that driven by death receptors is activated T-cell autonomous death (ACAD), which is determined by the ratio of anti and proapoptotic BCL-2 family members with the major role of a proapoptotic BIM protein. This type of cell death is known also as death by cytokine deprivation or by neglect [18]. Namely, the absence of appropriate survival signals induces BIM, which on the mitochondrial membrane can bind and neutralize the antiapoptotic BCL-2 or BCL-X_L (see below).

T-cells, similarly to other cells, can undergo Damage-Induced Cell Death (DICD), which is a cell response to DNA damage induced by both extracellular and intracellular insults, such as reactive oxygen species [13].

All three processes, although differentially regulated culminate in apoptotic cell death named also programmed cell death.

2 Apoptotic Pathways in T-cells

Apoptosis, or programmed cell death, is a fundamental process essential for both development and tissue homeostasis (reviewed in [21]). In the immune system, apoptosis plays a crucial role in selection of T-cell repertoire in the thymus, deletion of self-reactive T- and B-lymphocytes both in the central and peripheral lymphoid compartments, and in the killing of target cells by cytotoxic T-lymphocytes and natural killer cells [33]. Defects in apoptosis have been associated with a number of disease states, including autoimmunity and AIDS [48].

Cells undergoing apoptosis exhibit specific morphological changes, including membrane blebbing, cytoplasmic and chromatin condensation, DNA fragmentation, nuclear breakdown and assembly of membrane-enclosed vesicles termed apoptotic bodies, eventually subjected to phagocytosis [55]. The dying cells express "eat-me" signals, such as phosphatidyl serine, which allow the cells to be removed by phagocytosis.

Two major signaling pathways of apoptosis have been described: the extrinsic pathway induced by ligation of death receptors, and the intrinsic pathway comprised of the mitochondrial and endoplasmic reticulum pathways and induced by DNA damage, cytokine deprivation, gluccocorticoids or stress (Fig. 1). There is some crosstalk between these apoptotic pathways, but they lead to activation of different initiator caspases, which in turn activate common effector caspases 3, 6 and 7 [16, 26]. One of the terminal events of the apoptotic signaling pathways is activation of specific endonucleases cleaving DNA into oligunucleosomal fragments: Endo G and DFF/CAD. The latter is activated by effector caspases which cleave the DFF/CAD inhibitory protein [53]. The effector caspases cleave also a number of other important cellular proteins including actin, lamins, gasoline, plectin and others; their degradation in turn leads to the blebbing and formation of apoptotic bodies and final cell destruction.

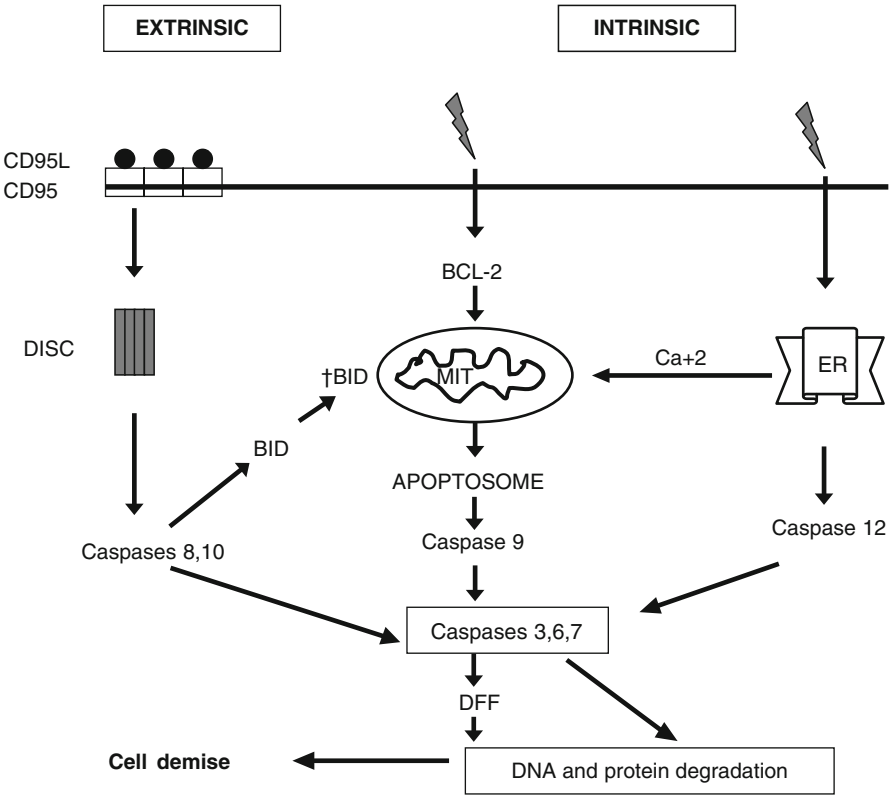

Fig. 1 The cross-talk of apoptotic pathways
The extrinsic pathway is mediated by interaction between death receptor and its ligand and leads to activation of initiator caspases 8, 10. The intrinsic pathway is comprised of the mitochondrial pathway (MIT) and the endoplasmic reticulum (ER) pathway. The mitochondrial pathway leads to activation of initiator caspase 9 and endoplasmic reticulum pathway to activation of initiator caspase 12. All initiator caspases activate effector caspases 3, 6 and 7 which in turn activates specific endonuclease (DFF) and depredate cellular proteins.

The intrinsic apoptotic pathway crucially depends on permeabilization of the outer mitochondrial membrane and mitochondria seem to be integrators of the many apoptotic signals coming from the outside and inside the cell. After receiving an apoptotic signal, mitochondria release a variety of molecules of which cytochrome c seems to be the most important one, which together with cytoplasmic apoptotic-protease-activating factor 1 (APAF1) forms the apoptosome. At the apoptosome initiator caspase 9 is activated. Crucial roles in determining the mitochondrial membrane permeability is controlled primarily by a balance between the antagonistic actions of the proapoptotic and antiapoptotic members of the BCL-2 family. Antiapoptotic members, such as BCL-2 and BCL-X_L possess so named BH1-4 domains. Proapoptotic BCL-2 family proteins comprise two subfamilies: the first including BAX, BAK and BOK, which have BH1-3 domains and the second including BH3-only members, such as BAD, BID, BIM and others. BH3- only proteins, like BIM can bind and neutralize the antiapoptotic BCL-2 or BCL-X_L. This in turn activates proapoptotic BAX or BAK which release cytochrome c from mitochondria.

The extrinsic apoptotic pathway is triggered by signals originating with cell-surface death receptors belonging to the TNF receptor (TNFR) superfamily that are activated by several ligands such as CD95L (also known as FasL), tumor necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL). Transduction of the apoptotic signal from the death receptors starts with the formation of a large protein complex at the cell membrane, known as the death inducing signaling complex—DISC. The CD95 DISC consists of trimerized CD95, the adaptor molecule FADD containing so called DD domain, procaspase 8a (also named FLICE), procaspase 8b, procaspase 10 and the cellular FLIP (cFLIP) protein. FLIP protein contains inactive caspase-like domain. Three isoforms of cFLIP are known, but only cFLIPs seem to exert an inhibitory effect on caspase 8 activation. Procaspases 8 and 10 as well as FADD and cFLIP contain DED domain which is required for DISC formation. Thus, formation of the DISC results in the assembly of procaspase 8 and procaspase 10 leading to their autoproteolytic activation. In some cells this signaling pathway suffices to induce executor caspases and eventual cell death (Type I cells). However, the level of CD95 DISC and of active caspase 8 may be too low (Type II cells) and the signal requires on additional amplification loop involving the cleavage of a BH-only BCL-2 family protein, BID, by caspase 8 to form truncated BID (tBID). tBID in turn aggregates BAX or BAK, which leads to mitochondria membrane permebilization and cytochrome c release [26].

3 Mechanism of Activation-Induced Cell Death

AICD is believed to be the major mechanism of elimination of T-cells during the termination phase of immune response. Following pathogen entry, T-cell activation *via* TCR and coreceptors induces their proliferation and clonal expansion. Pathogen killing by effector cells is followed by a severe decrease of T-cell proliferation and the beginning of T-cell elimination by death receptor-dependent apoptosis (Fig. 2)

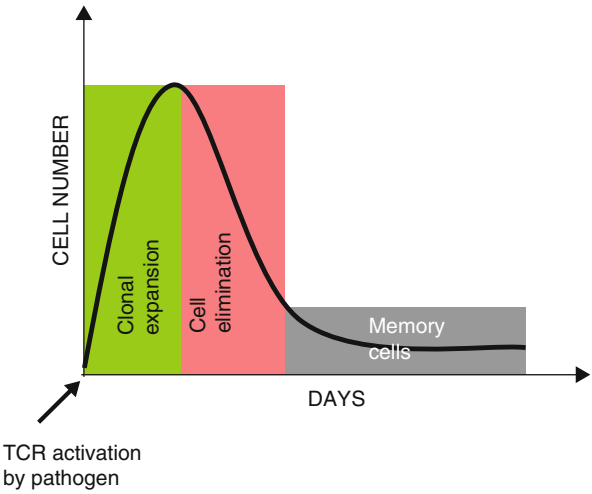

Fig. 2 Immune response Following patogen entry, TCR activation induces T-cell proliferation and clonal expansion. Pathogen killing by effector cells is followed by cell elimination by death receptor-driven apoptosis.

[22]. Upon stimulation of activated T-cells CD95L mRNA and protein expression on the surface of cell are rapidly induced. CD95L binds to the CD95 receptor on the same cell that express CD95L or on neighboring cells and triggers CD95-dependent apoptosis. The first type of CD95/CD95L interaction results in autocrine (suicide) and the second type in paracrine (fratricide) type of death [26].

Studies performed on murine and human T-cells suggested several transcription factors to be involved in activation of CD95L expression, such as Ap-1, NFAT, NFκB, c-Myc, Erg 1 & 3, and others. Cooperation between some of them is necessary for CD95 gene activation. Also, several tyrosine kinases known to be involved, such as PKC, Lck, ZAP-70 and MAPK, among others [14, 19].

An in vitro system of T-cell immune response believed to mimic the shutdown of immune response occurring in vivo has been developed by Krammer's group [24] and now this model is used, with some modifications by many investigators. Originally, freshly isolated primary human T-cells were activated with the non-specific mitogen-PHA for several hours (short–term activated), which respond to CD95 driven apoptosis resistance. However, after in vitro culture lasting for several days (long-term activated), the activated T-cells acquired sensitivity to death receptor-driven cell death, and IL-2 was found to be necessary for this sensitization [27, 43].

CD95 is not expressed on T-cells derived from cord blood or on naïve resting T-cells, but is rapidly up-regulated upon T-cell activation [23]. Although it was shown that long-term activated cells prone to AICD and short-term activated cells resistant to AICD express similar high amounts of CD95 [24]. This observation suggests that the signaling cascade downstream of CD95 must be modulated. Indeed, the cFLIP protein, and particularly its cFLIPs isoform, a potent inhibitor of CD95-mediated apoptosis, was found to be expressed at a high level in short- but not in long-term activated T-cells [2, 20]. Moreover, it was shown that inhibition of IL-2 production or signaling prevented down-regulation of FLIP protein levels and conferred resistance to CD95-mediated apoptosis on TCR-activated cells [2]. It cannot be

excluded that expression of antiapoptotic BCL-2 family members, such as BCL-2 and BCL-X_L, might provide additional protection against cell death at the level of mitochondria [3].

Overall, AICD regulation is rather complex and involves both extrinsic and intrinsic mechanisms of apoptosis; it is also tightly connected with survival signaling pathways triggered by TCR and cytokines [26] (Fig. 3).

TCR–mediated NFkB signaling is critical for cell survival (death resistance) by inducing prosurvival and antiapoptotic genes [3]. It has been postulated that the hematopoetic progenitor kinase HPK1-C mediates sensitivity towards AICD by suppression of NFκB activity [4].

4 Age-Related Alterations of Activation-Induced Cell Death of Human T-cells

The aging of the immune system, termed immunosenescence involves several components which lead to its decreased functionality contributing to the morbidity and mortality of elderly people. The main aspects of immunosenescence are: (i) the

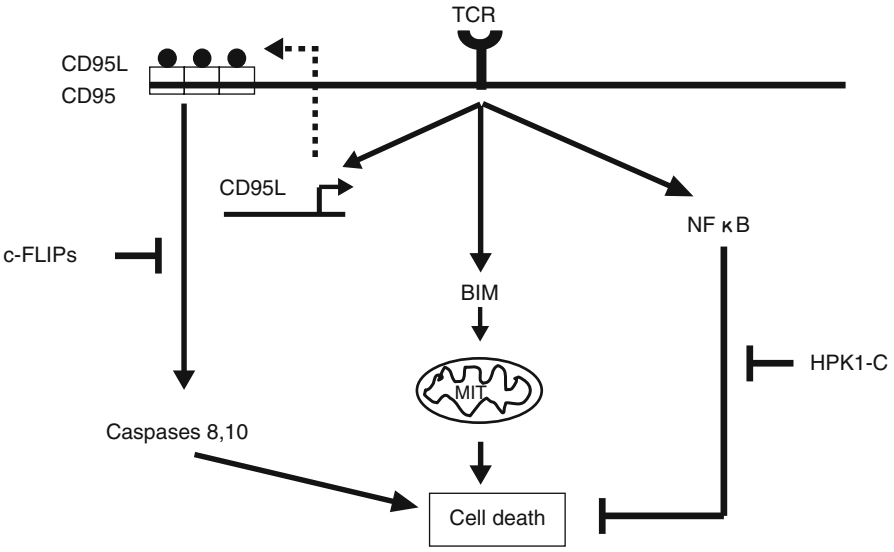

Fig. 3 TCR-mediated signals to cell death and survival
Stimulation of the TCR receptor can lead to CD95L transcription and engagement of CD95, thus activation of the extrinsic apoptotic pathway. TCR stimulation can also cause activation of the intrinsic apoptotic pathway via BH3-only BCL-2 family BIM. The extrinsic death pathway can be connected to the intrinsic pathway by caspase 8-mediated cleavage of the BH3-only BCL-2 family member BID towards truncated BID (tBID). TCR-mediated NFκB signaling is critical for cell survival and can be blocked by HPK1-C. On the other hand, apoptosis can be blocked by c-FLIPs.

involution of the thymus and exhaustion of naïve T-cells; (ii) the diminution of the T-cell repertoire and accumulation of oligoclonal expansions (megaclones) of memory/effector cells; and (iii) a chronic inflammatory state called inflamm-aging [8].

T-cell senescence in humans involves alterations similar to those observed in other cell types, such as changes in functions, cessation of cell proliferation due to shortening of telomeres and a changed propensity to undergo apoptosis. The senescence is seen primarily in the CD8+ T-cell population but it also occurs in CD4+ T-cells [11, 50].

Alterations of apoptosis in T-cells from aged individuals have been reported by many investigators, however, a consensus is still lacking in this matter especially since many studies were only correlative. Some authors show that aging is associated with increased apoptosis of T-cells, whereas others report the opposite (reviewed in [15, 19, 29]). These apparent discrepancies might be due to differences in the stimuli investigated, the phenotype of the cells as well as the general experimental approach. Also, some experiments were performed on lymphocytes undergoing in vitro replicative senescence [45] while others on cells derived from donors of different ages [36]. According to our results, replicative senescence in vitro only partially reflects the in vivo process [5-7].

Moreover, there is profound confusion in the literature concerning the term AICD, partially due to some overlapping of dead receptor-driven and mitochondria-mediated apoptosis in this process. Second, the question is whether the propensity of activated T-cells to undergo apoptosis upon death receptors activation (treatment with CD95L or anti CD95 mAb) can be considered as AICD? Taking into account that AICD is undergoing *via* engagement of death receptors this can help in understanding this process. (Death receptor-driven apoptosis is the subject of another chapter).

However, we must keep in mind that according to classical definition, AICD is induced by religation of TCR on activated cells [25]. Finally, it should be also remembered that AICD is not the only mechanism of T-cell death during shutdown of the immune response [47].

Generally, the literature guides us to three possibilities concerning AICD in the aging process. The first is that **T-cell susceptibility to AICD is increased**.

Despite the induction of CD95L, the main indictor of the cell ability to undergo AICD is the expression of CD95 receptor. While T-cells from cord blood are CD95-negative, the proportion of CD95-positive cells are growing with age [12, 41]. Moreover, Aggarwal and Gupta [1] reported on increased expression of CD95 mRNA in the elderly in comparison with young subjects. Fagnoni et al. [12] postulated the CD95-negative cells disappearing with age are unprimed, naïve cells. We also found a dramatic decrease in percentage of CD95-negative cells with age, from virtually 100% (not shown) in the cord blood to almost undetectable in peripheral blood of centenarians (Fig. 4). However, PBMC cultures from young and old individuals alike show an increase in CD95 expression upon activation, the cells from old individuals reaching even higher CD95 levels than those from young individuals [31, 37, 40]. Therefore, T-cells from the elderly are able to respond to activation with CD95 engagement. Moreover, some studies of T-cells from elderly subjects do appear to show a correlation between the increased CD95 expression and increased activation-induced

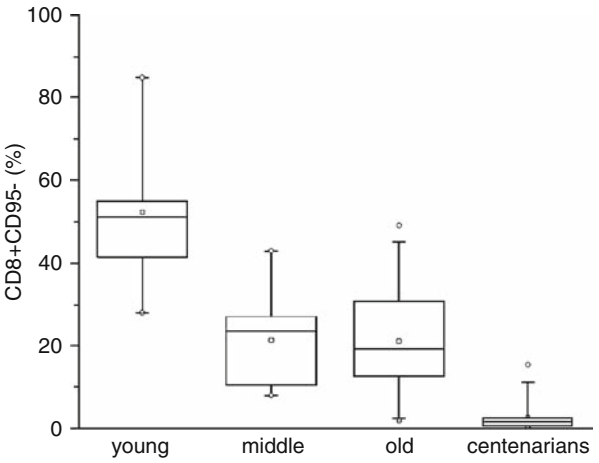

Fig. 4 Fraction of CD95- cells in peripheral blood of donors at different ages Young (aged 25–35, N=16), middle (aged 45–55, N=11), old (aged 65, N=37), centenarians (N=32).

cell death [30, 37, 40]. Lechner et al. [30] observed no differences in the incidence of apoptosis in response to activation between PBMC from young and old subjects. However, under long-term cultivation, namely at replicative senescence in vitro, an increase of AICD was observed and it was more pronounced in T-cell populations from old than from young individuals. Phelouzat et al. [36] reported a greater depletion of T-cells upon stimulation in old than in young PBMC which was shown to be due to apoptosis. Similar results were obtained by Potestio et al. [40]. Schindowski et al. [42] described a slight but statistically significant increase of AICD in elderly in comparison to young individuals. Pawelec et al. [35] reported on increased susceptibility to AICD in a late-passage in comparison to an early-passage CD4+ cultured T-cell clone. Greater CD95-induced apoptosis was found in anti-CD3 stimulated CD4+ than in CD8+ cells derived from healthy donors, and both CD4+ and CD8+ T-cells from the elderly were more sensitive than those from young individuals [1].

The presented so far data indicate that activated T-cells from the elderly are more susceptible to undergoing AICD than cells from younger subjects, moreover, the population of CD4+ cells is more sensitive to cell death then the CD8+ cells.

Based on those studies as well as the results emerging from studies on mice, Ginaldi proposed recently an increased propensity to AICD as a hallmark of aging [13]. However, the literature also shows evidence in favor of other possibilities, namely that **T-cell susceptibility to AICD is unchanged or even decreased with age.** Pinti et al. [38], similarly to others reported on increasing number of CD95-positive cells and an increased level of Fas mRNA with age. Although the reverse trend was observed in the case of FasL in resting cells, the amount of FasL produced by lymphocytes upon activation with anti-CD3 was the same irrespective of the age of donors (young, middle-aged, centenarians). Also AICD level was the same in T-cells from all three groups. This is in agreement with our results showing no differences in AICD levels in T-lymphocytes derived from young in comparison to old donors. Following a slightly modified classical protocol described by Krammer's group [24], PBMC cells were stimulated with PHA for 72 h and then cultured in the presence of IL-2

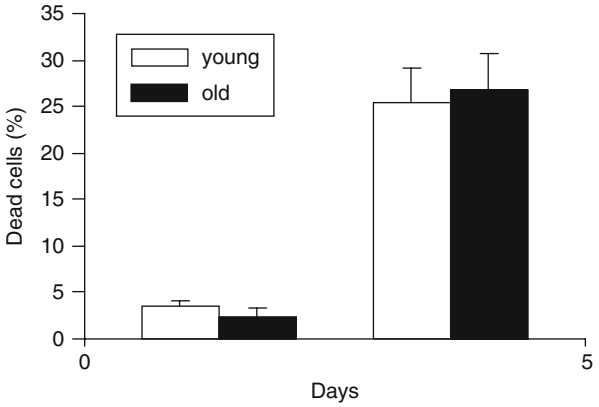

Fig. 5 AICD of T-cells derived from young (aged 25-31, N = 4) and old (aged 65, N = 4) donors. PBMC cells were stimulated with PHA for 72 h and then cultured in the presence of IL-2.

(Fig. 5). However, this protocol does not correctly reflect the situation in vivo, as the production of IL-2 is severely diminished with age. Similar results, namely the same level of AICD in young and old subjects, were obtained by Herndon et al. [17]. However, more recent results from this laboratory indicated for decreased AICD in donors aged 70–85 years in comparison with younger ones (25–65-year old), but also in comparison with nonagenarians. Thus from this results it is difficult to conclude whether AICD is really diminished with the age [19]. Whereas, Donnini et al. clearly showed that the AICD level analyzed in CD4+ cell subsets, namely naïve (CD62L+CD95-) and memory (CD62L-CD95+), did not correlate with the age [10].

Indirect, but convincing arguments of reduced AICD in elderly come from experiments on the role of lipid rafts in immunosenescence. Lipid rafts are involved in many processes, but mainly in signal transduction in T-cells, which is obviously impaired in the elderly [28, 29]. It was shown that disruption of lipid rafts reduced the sensitivity to Fas-mediated apoptosis after TCR restimulation of CD4+ cells. Thus, the redistribution of Fas and other tumor necrosis factor family receptors into and out of lipid rafts may dynamically regulate the efficiency and outcomes of signaling by these receptors [32]. Larbi et al. [29] reported a decreased expression of Fas and FasL in old in comparison with young donors. Expression of Fas was diminished and expression of FasL was completely abolished in stimulated lymphocytes after disruption of lipid rafts. It was shown that activation of lipid rafts was possible only upon ligation of both TCR and CD28, implying that CD28 might be critical in the signal transduction leading to AICD.

5 The Role of CD28 Coreceptor in Age-Dependent AICD of T-cells

Senescence effects numerous changes in the phenotype and the functioning of T-cells. Lifelong and chronic antigenic load may represent the major driving force of immunosenescence due to reducing the number of naïve antigen-nonexperienced

cells and their replacement by expanded clones of antigen-experienced effector and memory T-cells with late differentiated phenotype. The thymus releases fewer naïve cells with age and those T-cells remaining, especially the CD8+subset, show increased oligoclonality with age [51].

The recognition of MHC-bound antigen by TCR is a low-affinity interaction unable to sustain activation of T-cells; productive activation requires costimulation with CD28 which serves as an amplifier of the TCR signal [50]. By activating Akt, CD28 acts as a typical transducer of the prosurvival pathway [19].

It is known that T-cell activation leads to CD28 down-regulation. Indeed, various models of T-cell replicative senescence show that subsequent rounds of cell divisions eventually lead to accumulation of CD28-negative cells, which are the progeny of CD28-positive ones [7, 11, 39]. We showed a gradual replacement of CD8+CD28+ cells by CD28- cells in long-term cultures both in the cord blood and in the peripheral blood of donors of different age, including centenarians [7]. It was also shown that purified human CD28+ T-cells progressively lose CD28 during each successive stimulation, with CD8+ T-cells losing CD28 more rapidly than CD4+ cells [50]. Also in vivo the accumulation with age of CD8+CD28- and to lesser extent CD4+CD28- is observed [7, 11, 12, 19, 50, 54]. CD28-negative cells are highly oligoclonal and have very short telomeres [50]. It is believed that they are unable to proliferate, however, we found that this is only true in the case of cells undergoing replicative senescence in vitro, but not for those aging in vivo [7].

As they accumulate progressively through life and large clones persist for years, CD28-negative cells are considered to be resistant to AICD. Indeed, Posnett et al. [39] demonstrated that CD8+CD28- cells activated with a superantigen were less susceptible to apoptosis than their CD8+CD28+ counterparts. Spaulding et al. [45] showed that T-cells reactivated after achieving in vitro the state of replicative senescence acquired resistance do apoptosis induced with different stimuli, including antiCD3 and antiFas.

Many of the CD8+ and CD28- expanded clones seem to result from previous infections by persistent viruses, especially CMV and to lesser extent, EBV and other herpesviruses. These are considered dysfunctional, “anergic” cells possibly at least partly due to apoptosis resistance, however a direct proof of their AICD resistance is lacking [51]. Thus it seems that accumulation with age of long-living CD8+CD28- cells can actually be explained by their relative resistance to AICD. Also CD4+CD28- cells, unlike their CD28+ counterparts, were shown to be protected from AICD due to high expression of cFLIP [49].

On the other hand, the data showing quite opposite correlation between CD28 and AICD can not be neglected. We showed no differences between CD28+ and CD28- in susceptibility to undergo AICD [6], but there are results providing evidences that maintenance of CD28 expression on T-cells may be even crucial for prevention of Fas-mediated apoptosis during the course of antigen engagement. Indeed, it was documented that within a superantigen-activated T-cell population, cells which were sensitive to Fas ligation were characterized by low CD28 expression prior to treatment with Fas [52]. It was also shown that CD28-mediated signaling increases expression of antiapoptotic BCL-X_L and thereby promotes survival

implying antiapoptotic activity of CD28. Indeed, Krammer's group reported that activated and cultured in the presence of IL-2 T-cells (undergoing AICD) when co-stimulated by CD28 showed, besides strong up-regulation of BCL-X_L, down-regulation of CD95L mRNA and strong up-regulation of cFLIPs [23]. In agreement with these results are data presented by others showing that low CD28 expression predispose to CD95L mediated apoptosis in activated T-cells and CD28 ligation protects from apoptosis [9].

6 Concluding Remarks

Immunosenscence is believed to be driven by thymus involution, continuous pathogen load and common damaging insults. This leads to the shrinkage of T-cell repertoire due to the reduction of naïve cells and accumulation of oligoclonal CD8+ and, to a lesser extent, CD4+ cells, displaying a highly differentiated and senescent phenotype with diminished functioning. Activation-Induced Cell Death plays a crucial role in the proper function of the immune system by elimination of expanded cells at the end of immune response. This logically implies a crucial role of AICD in immunosenescence. Indeed, some data published so far indicates AICD changes in the elderly. Nonetheless, this observations are inconclusive, some showing an increased some a decreased propensity of T-cells to AICD in the elderly. There are also reports of unchanged AICD with age. This apparent controversy probably stems from different experimental approaches and highly fragmentary data, especially concerning human studies. Systematic and comprehensive studies are still needed for a conclusive elucidation of the role of AICD in human aging.

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CD8 Clonal Expansions in Mice: An Age-associated Alteration of CD8 Memory T-cells

Eric T. Clambey, John W. Kappler and Philippa Marrack

Contents

P. Marrack (✉) · E. T. Clambey · J. W. Kappler
Integrated Department of Immunology
Howard Hughes Medical Institute
National Jewish Research & Medical Center
Departments of Medicine, Pharmacology
Biochemistry and Molecular Genetics
University of Colorado Health Sciences Center
Denver, CO 80206, USA
Telephone: +1 303 398 1322
Fax: +1 303 270 2166
E-mail: marrackp@njc.org

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Abbreviations

CFSE	5-(and -6) Carboxyfluorescein diacetate succinimidyl ester
EBV	Epstein-Barr virus
HCMV	Human cytomegalovirus
IFN-γ	Interferon gamma
IL	Interleukin
IL-2Rβ	Interleukin-2 receptor, beta chain
IL-7Rα	Interleukin-7 receptor, alpha chain
KLRG1	Killer cell lectin-like receptor G1
LCMV	Lymphocytic choriomeningitis virus
LIP	Lymphopenia-induced proliferation
MHC	Major histocompatibility complex
MP	Memory phenotype
PD-1	Programmed death-1
PMA	Phorbol 12-myristate 13-acetate
SPF	Specific-pathogen free
TCE	T-cell clonal expansion
TCR	T-cell receptor
T_{CM}	Central memory T-cell
T_{EM}	Effector memory T-cell
TRAF	Tumor necrosis factor receptor associated factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

Abstract: Aging is associated with a variety of perturbations in the immune system. One frequent alteration is a significant skewing of the CD8 T-cell repertoire. This alteration manifests as a clonal expansion of CD8 memory T cells, which in some cases can occupy the majority of the CD8 T-cell pool. CD8 clonal expansions are associated with impaired immunity in the elderly. Although CD8 clonal expansions are commonly found in aging humans and mice, the etiology of this phenomenon is unknown. Here, we describe our current understanding of CD8 clonal expansions as it relates to the current state of knowledge about CD8 T-cell memory. In addition, we discuss the heterogeneity observed between different types of clonal expansions in mice, and how distinct factors may influence both the development and properties of clonal expansions in the aging individual.

Keywords: Ageing • CD8 clonal expansion • CD8 memory T-cell • Homeostasis • TCE

1 T-cells, TCR Diversity and the Phenomenon of CD8 Clonal Expansions

A hallmark of the adaptive immune system is its capacity to respond to a myriad of different challenges. One way that individuals are able to respond to diverse pathogens is through the generation of a highly diverse T-cell repertoire, in which each T-cell expresses a slightly different T-cell receptor (TCR). Each TCR is created through a process of gene rearrangement of TCR gene segments, followed by further diversification methods. Notably, each distinct TCR recognizes a slightly different

combination of a short peptide (referred to as antigen) presented in the context of a major histocompatibility complex (MHC) molecule. Once a T-cell (and TCR) encounters its correct antigen and the appropriate stimulatory conditions, the T-cell can undergo a series of steps, including activation, proliferation, and the acquisition of effector functions through which the T-cell mediates its protective effects. CD8+ T-cells recognize antigen in the context of MHC Class I molecules and respond to, and control, a variety of intracellular infections (such as bacteria and viruses).

While young, healthy individuals possess a diverse CD8 T-cell pool, many aged individuals develop significant perturbations in the repertoire of TCR specificities. In these individuals, a single CD8 T-cell achieves a competitive advantage relative to its neighbors and comes to dominate the entire CD8 T-cell pool, a phenomenon referred to as CD8 T-cell clonal expansions (or TCEs). This phenomenon is of significant interest for many reasons. First, it is a common age-associated alteration to the immune system. Second, it results in a significant perturbation to the normally diverse CD8 T-cell repertoire. Third, it has been associated with impaired immunity in the aged. Fourth, it represents a significant breakdown in the normal homeostatic mechanisms that regulate CD8 T-cell survival and proliferation.

This chapter will focus on the biology of CD8 clonal expansions in mice, with only brief discussion of clonal expansions in humans. It should be noted that while there are some differences between clonal expansions in mice and in humans (for further discussion see [24]), CD8 clonal expansions in both species are characterized by the selective outgrowth of a specific subtype of CD8 T-cell, the CD8 memory T-cell. In order to understand the properties of clonal expansions, it is essential to have basic information about CD8 memory T-cells, their development, regulation, and biological properties.

2 CD8 Memory T-cell Differentiation

2.1 Memory T-cell Differentiation Following an Acute Exposure to Antigen

Following TCR stimulation by antigen, a naïve CD8 T-cell initiates a program of activation, proliferation and differentiation (Fig. 1a) [56]. The resulting CD8 T-cell response is characterized by multiple phases: i) proliferation and expansion of antigen-specific CD8 T-cells, during which these cells acquire a variety of effector functions (such as cytokine secretion and the capacity to kill target cells expressing the appropriate antigen), ii) a period of contraction during which 90–95% of the total number of antigen-specific CD8 T-cells undergo apoptosis, and iii) a period of further differentiation during which the remaining 5–10% of antigen-specific CD8 T-cells acquire additional phenotypic changes, to ultimately become a CD8 memory T-cell (Fig. 1a). During the expansion phase, antigen-specific CD8 T-cells can occupy a massive fraction of the CD8 T-cell pool (e.g., during acute lymphocytic choriomeningitis virus (LCMV) infection, at least 80% of the CD8 T-cells in the spleen are specific for LCMV [84]).

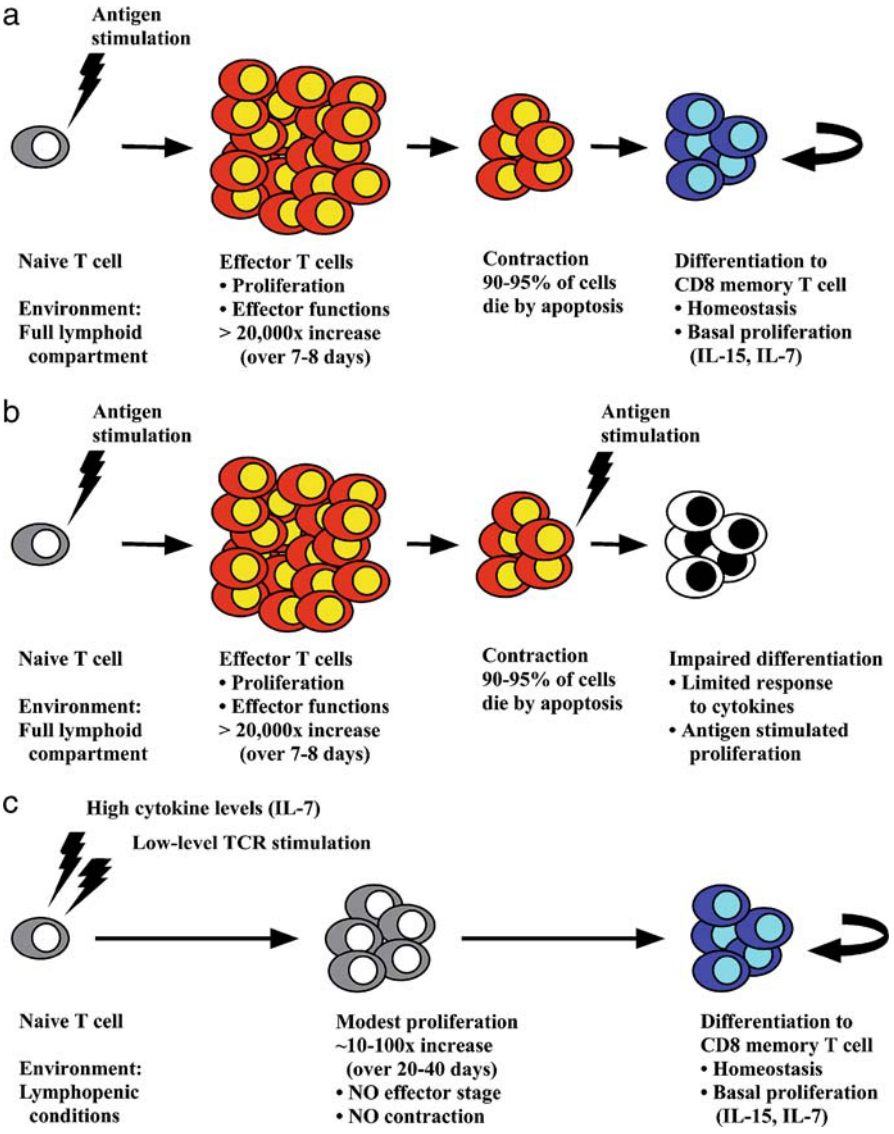

Fig. 1 CD8 memory T-cell differentiation in various contexts
The dynamics of an antigen-specific CD8 T-cell response in individuals possessing a full lymphocyte compartment following either transient, acute antigen exposure (panel a) or prolonged, persistent antigen exposure (panel b). CD8 memory T-cells can also develop when naïve T-cells are placed in a lymphopenic environment, devoid of other lymphocytes (panel c). The different stages of CD8 T-cell differentiation are indicated as follows: naïve (gray cytoplasm, white nucleus), effector cell (red cytoplasm, yellow nucleus), memory cell (deep blue cytoplasm, light blue nucleus). In the case of persistent antigen, there is impaired memory cell differentiation, as indicated by white cytoplasm, black nucleus. Stages of differentiation and factors that influence CD8 T-cell response are discussed further in text. Diagram indicates relative, not absolute abundance of CD8 T-cells at each stage.

CD8 memory T-cells differ from naïve CD8 T-cells in many ways. In contrast to naïve CD8 T-cells, CD8 memory T-cells have an accelerated response to antigen stimulation, are found at many sites in the body and are maintained at very constant levels for months to years following initial antigen exposure (a process facilitated by cytokines). The self-renewing capacity of CD8 memory T-cells can occur in the absence of continued antigen exposure, providing an antigen-independent mechanism of CD8 memory T-cell maintenance. The combination of these characteristics, as well as the increased frequency of antigen-specific CD8 T-cells in antigen-exposed individuals, means that individuals who develop a CD8 memory T-cell response against an antigen will have a rapid, robust response upon antigen reexposure. In many contexts, CD8 memory T-cells provide an important mechanism of immunological protection, or immunity to reinfection [56, 140].

In the context of CD8 clonal expansions, one particularly important property of CD8 memory T-cells is their steady, slow rate of proliferation in the uninfected animal. The long-term proliferation and survival of CD8 memory T-cells is heavily dependent on the cytokines interleukin (IL)-7 and IL-15 [7, 37, 72, 116, 118, 125]. CD8 memory T-cells receive these cytokine cues through expression of the IL-7 receptor alpha chain (IL-7Rα, CD127), the IL-2 receptor beta chain (IL-2Rβ, CD122), and the common gamma chain cytokine receptor. As discussed below, CD8 clonal expansions have alterations in their capacity to respond to these cytokines.

2.2 Memory T-cell Subsets

Though CD8 memory T-cells arise following antigen stimulation, not all CD8 memory T-cells have the same properties. CD8 memory T-cells are most frequently categorized as either central memory (T_{CM}) or effector memory (T_{EM}) cells [113]. While both these subsets of memory cells express cell surface receptors thought to be typical of CD8 memory T-cells (e.g. in the mouse, $CD44^{high}CD127^{high}CD122^{high}$), these two subsets differ in their expression of L-selectin (CD62L) and the chemokine receptor, CCR7, two proteins that promote trafficking to peripheral lymph nodes. Central memory T-cells are CD62L+ CCR7+, and are found in blood, spleen, and lymph nodes. In contrast, effector memory T-cells do not express CD62L or CCR7, and are found in blood, spleen, and nonlymphoid tissues. T_{EM} cells are generally absent from lymph nodes, except in situations of ongoing inflammation [38, 85].

The precise factors that influence whether a CD8 memory T-cell becomes a central or an effector memory cell remain contentious. One factor that might influence this balance is the magnitude of the initial antigenic stimulus [132]. It should be noted, however, that at this time it is debatable whether the T_{CM} and T_{EM} subdivisions of CD8 memory T-cells represent true independent cell fates, are capable of interconversion, or whether their differences reflect in part the impact of local environments on the phenotype and function of a CD8 memory T-cell (for further discussion see [76]). Regardless of the precise development details of T_{CM} and T_{EM} cells, local tissue environments can significantly influence the phenotype and properties of a CD8 memory T-cell [67, 83, 86].

Following the identification of T_{CM} and T_{EM} subsets of CD8 memory T-cells, further subsets of memory cells have been identified. For example, the IL-7Rα (CD127) was identified as a marker to identify activated CD8 T-cells that gave rise to long-lived memory cells [46, 55]. Subsequent work has shown that the usefulness of this marker varies depending on the experimental system [73], and that IL-7 plays an important but perhaps not instructive role in the development of CD8 memory T-cells [15, 40, 66, 123]. A recent study also identified expression of CD8αα homodimers as a potential marker for CD8 memory T-cell precursors [80], although the significance of this observation remains contentious [21, 135, 143]. Additional subtypes of CD8 memory T-cells, differing by various criteria (e.g., ability to divide in the absence of antigen, tissue distribution, and capacity to respond upon antigen rechallenge) have also been identified [12, 44, 109]. Despite the identification of these various subsets, a major unanswered question is the interrelationship between different types of memory T-cells and the factors that drive these distinct phenotypes and properties.

It is worth noting that in some studies of CD8 memory T-cells, cells have the phenotype of a memory T-cell (in the mouse, typically defined as $CD44^{high}$), but the precise antigen reactivity and origin of this memory cell is poorly defined. These cells are often referred to as CD8 memory phenotype (MP) T-cells. Some CD8 MP T-cells probably result from conditions of lymphopenia (discussed below).

2.3 CD8 Memory T-cell Differentiation in the Context of Chronic Infection

The above discussion of CD8 memory T-cell differentiation focused on this process following an acute, transient infection. It is important to note, however, that CD8 T-cells are highly attuned to external cues, and that the process of CD8 memory T-cell differentiation can be significantly influenced by the nature of the eliciting infection (e.g., [2]). In addition, the phenotypes and properties of a CD8 T-cell can vary between CD8 T-cells responding to different epitopes within the same pathogen [45, 121]. These different outcomes likely reflect differences in the patterns of antigen expression at various stages of infection.

The most dramatic perturbations to CD8 memory T-cell differentiation occur in situations of chronic infection (e.g., certain strains of LCMV) that are characterized by a prolonged, high pathogen burden. In these situations, CD8 T-cells develop an altered state of "memory" in which the resulting CD8 T-cells remain actively dependent on persistent antigen and TCR engagement for their survival (Fig. 1b). These cells express reduced levels of cytokine receptors for IL-7 and IL-15 (IL-7Rα and IL-2Rβ, respectively), and do not achieve antigen-independent survival and proliferation [120, 131]. In addition, these cells can express sustained levels of inhibitory receptors such as programmed death-1 (PD-1), which can actively impair the capacities of a CD8 T-cell [6]. Situations of chronic infection can also result in the continual recruitment of naïve CD8 T-cells into the CD8 memory pool [130].

Based on the above differences, it is worth considering whether the CD8 "memory" T-cells that result during a chronic infection are true CD8 memory T-cells or if they instead exist in an altered state of "quasi-memory". For the purpose of this chapter, we will refer to CD8 memory T-cells that develop in the context of chronic infection as antigen-dependent CD8 memory T-cells (referring to their continued requirement for antigen to survive). This is in contrast to CD8 memory T-cells that arise following an acute infection, which we will refer to as antigen-independent CD8 memory T-cells (referring to their capacity to survive in the absence of antigen).

2.4 An Alternate Way to become a CD8 Memory T-cell: Lymphopenia-induced Proliferation

While CD8 memory T-cells have been traditionally studied in individuals following exposure to a variety of antigens, there is an alternate way for a naïve CD8 T-cell to become a CD8 memory T-cell. This phenomenon occurs in individuals characterized by a state of severely reduced lymphocyte numbers, a condition known as lymphopenia. Lymphopenia is observed in various conditions, including individuals exposed to high dose irradiation or chemotherapy, as well as in neonates [75, 92]. In mice, genetic models of lymphopenia are also available (such as mice completely devoid of T-cells).

The observation that lymphopenia could promote the generation of CD8 memory T-cells was made by multiple groups who transferred naïve, antigen-specific CD8 T-cells into lymphopenic mice (whether irradiated or genetically deficient) (reviewed in [51]). In these studies, naïve CD8 T-cells began to proliferate once placed in the lymphopenic environment, a process referred to as either homeostatic proliferation or lymphopenia-induced proliferation (LIP). In addition to proliferating, however, these CD8 T-cells also acquired many of the characteristics associated with a CD8 memory T-cell [22, 35, 64, 93, 96]. For clarity, these cells will subsequently be referred to lymphopenia-induced proliferation (LIP) CD8 memory T-cells.

At this time, it is unknown whether LIP CD8 memory T-cells and antigen-elicited CD8 memory T-cells are identical. There are clear differences in the generation of these two cell types (compare Fig. 1a and 1c). First, LIP CD8 memory T-cells do not go through a stage of acute activation (e.g., LIP CD8 memory T-cells do not express various early activation markers), in contrast to antigen-elicited CD8 T-cells [22, 93]. In addition, LIP memory cells undergo a much more modest proliferation than antigen-elicited memory T-cells, and have no significant contraction phase [22, 93]. Despite these differences, LIP memory cells do have a transcriptional profile that is similar to that of antigen-elicited CD8 memory T-cells [36], and these cells are capable of mediating a protective response against secondary infection [39].

The observation that a naïve CD8 T-cell can become a memory cell in the absence of strong antigenic stimulation indicates that there is at least one alternate way for a naïve cell to become a memory T-cell (Fig. 1c). While lymphopenia-induced proliferation

can be promoted by TCR stimulation by low affinity ligands [30, 34, 64], this phenomenon is also driven by the high levels of unconsumed cytokines (particularly IL-7) present in an environment that is almost devoid of neighboring lymphocytes (reviewed by [51, 124]). At this time, the precise contribution of LIP memory T-cells to the complete CD8 memory T-cell repertoire is unclear. Nonetheless, given some of the factors that influence the development of CD8 clonal expansions (described below), lymphopenia-induced proliferation and memory differentiation may contribute to at least part of this age-associated phenomenon.

3 The Regulation of CD8 Memory T-cell Homeostasis

3.1 The Role of IL-7 and IL-15

As previously alluded to, the regulation of CD8 memory T-cell proliferation and survival is heavily influenced by extracellular factors. The cytokines IL-7 and IL-15 are the best-characterized extracellular proteins that promote the survival and proliferation of CD8 memory T-cells [7, 37, 72, 116, 118, 125, 142]. Both of these cytokines belong to the common gamma chain (γ_c) family of cytokines.

In general, the functions of IL-7 and IL-15 are thought to be compartmentalized, such that IL-7 primarily provides survival signals whereas IL-15 provides proliferative signals (reviewed in [124]). Although excess IL-7 can overcome a deficiency in IL-15 [65], the mechanism by which these two cytokines are perceived differs significantly. IL-7 is present in a secreted, soluble form. In contrast, IL-15 appears to be retained on the cell surface of certain cells, requiring direct cell contact of the CD8 T-cell with an IL-15 presenting cell in order to receive an IL-15 signal [17, 27, 114, 117]. While IL-7 and IL-15 can function alone, their effects can also be influenced by other cytokines. For example, IL-21 can synergize with IL-15 to promote proliferation of CD8 memory T-cells in vitro [141].

Given the central role of IL-7 and IL-15 in promoting CD8 memory T-cell homeostasis, the levels of these cytokines are tightly controlled and for good reasons. Limited cytokine expression appears to be important in limiting excessive proliferation; transgenic mice that express excessive amounts of IL-15 can develop a fatal leukemia [32]. Cytokine signals are also subject to additional regulation. For example, IL-7 can downregulate expression of its own receptor, IL-7Rα [101].

3.2 The Role of Other Cytokines, Cell Surface Receptors and Cells

In addition to IL-7 and IL-15, other cytokines also influence the homeostasis of CD8 memory T-cells. For example, IL-2 appears to be critical for CD8 memory

T-cells to robustly proliferate upon antigen reexposure [136]. In contrast, transforming growth factor beta (TGF-β) appears to limit the rate of proliferation of CD8 memory T-cells, possibly through antagonism of IL-15 signals [79]. High levels of IL-10 can also impair the appropriate formation of CD8 memory T-cells, as revealed by studies of chronic LCMV infection [14, 28].

Various cell surface proteins of the immunoglobulin and tumor necrosis family (TNF) families can also influence the magnitude and homeostasis of CD8 memory T-cells. Mice deficient in the B- and T-lymphocyte attenuator (BTLA), an immunoglobulin superfamily member, have an increased number of CD8 MP T-cells and a higher rate of homeostatic proliferation, indicating that BTLA limits the magnitude of CD8 memory T-cells [69]. In contrast, mice deficient in the TNF receptor ligand 4-1BBL have impaired CD8 memory, suggesting a positive role for 4-1BB signaling in the formation of a robust CD8 memory T-cell response (reviewed in [111]). Similar data indicate a positive role for CD27 and OX40 in promoting CD8 memory T-cell responses [42, 43]. Notably, some of the effects of these proteins may be directly regulated by cytokine cues elicited by IL-15 [107].

While many of the above cues influence the long-term maintenance of CD8 memory T-cells, initial signals received during T-cell activation can also heavily influence the differentiation of a naïve CD8 T-cell to a CD8 memory T-cell. One example of this regulation is the observation that inflammation can prolong the time required for CD8 memory T-cell differentiation [41]. As such, CD8 T-cells activated in a context of minimal inflammation become memory T-cells more rapidly (e.g., following immunization with antigen-pulsed dendritic cells) [5]. At least part of this effect is mediated by the effect of inflammatory cytokines, such as interferon gamma (IFN-γ), on the responding CD8 T-cell [5]. It is worth noting, however, that the effects of IFN-γ on the immune system are pleiotropic, and in some contexts, IFN-γ can promote an optimal CD8 memory T-cell response [133, 134]. IL-12 and type I interferons can also promote optimal CD8 T-cell activation and CD8 memory responses [68, 88].

The properties of CD8 memory T-cells are also heavily influenced by the presence or absence of CD4+ T-cells. Over the past few years, there has been an increasing appreciation that CD8 memory T-cells generated in the absence of CD4 T-cell help can be compromised in various ways (reviewed in [8]). At least part of the defect observed in CD8 memory T-cells that do not receive CD4 T-cell help may be due to tumor-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induced apoptosis of CD8 memory T-cells upon antigen re-exposure [39, 52]. However, additional mechanisms are also likely involved in CD4 T-cell optimization of CD8 memory T-cell responses [4, 95].

Finally, the properties of CD8 T-cells can be influenced by the frequency of antigen-specific T-cells that participate in a response, as well as subsequent antigenic exposure. Studies analyzing the response of TCR transgenic CD8 T-cells, in which each CD8 T-cell expresses the identical TCR as its neighbors, have revealed that an artificially elevated number of identical antigen-specific T-cells (achieved by adoptive transfer of a high number of TCR transgenic CD8 T-cells) results in CD8 T-cells with distinct properties not observed during an endogenous CD8 T-cell response

[3, 59, 82]. At this time, it is unclear whether this observation reflects an experimental artifact, or whether it reflects some basic physiological regulation observed in certain conditions of CD8 T-cell responses. Although CD8 memory T-cells can be maintained in an antigen-independent manner, subsequent antigen exposures can influence the TCR specificities of CD8 memory T-cells that are maintained [119].

3.3 The Influence of Intracellular Factors

While the properties of CD8 memory T-cells are well defined, the intracellular factors that coordinate these changes remain poorly characterized. CD8 memory T-cells are clearly characterized by a wide variety of transcriptional changes [54], as well as changes in chromatin modifications relative to naïve CD8 T-cells [31, 61, 95]. While there is no identified master regulator for the development of CD8 memory T-cells, there have been an increasing number of transcription factors that either facilitate differentiation to, or the properties of, CD8 memory T-cells. These include Bcl-6 [47], STAT5 [16, 58], eomesodermin and T-bet [49, 103], Bcl-6b/BAZF [81], c-myc [9], MeCP2 [60], and Id2 [19]. At this time, the precise molecular targets of these transcription factors and their contribution to CD8 memory T-cell development remain largely undefined.

Intracellular proteins that influence the proliferation and survival of CD8 T-cells can also impact the development of CD8 memory. The suppressor of cytokine signaling (SOCS) family of proteins is known to inhibit various cytokine signals [1]. In particular, SOCS1 is an important regulator of CD8 T-cell responses to cytokine signals by IL-7 and IL-15, and deficiency of this molecule results in an increased number of CD8 MP T-cells [23, 26, 48].

Regarding proteins that regulate cell survival, the proapoptotic Bcl2-family member, Bim, appears to limit the number of cells entering the CD8 memory T-cell pool [138]. Signal transduction through tumor necrosis factor receptor associated factor (TRAF) 1 is one mechanism that may regulate levels of Bim protein during a CD8 T-cell response [112]. The optimal development of CD8 memory also depends on appropriate protection of CD8 T-cells against internal damage from cytotoxic proteins expressed by CD8 T-cells (e.g. granzymes, cathepsins), something which can be mediated by various serine protease inhibitors expressed in CD8 T-cells [78, 104].

4 The Discovery of CD8 Clonal Expansions

Following the discovery of the T-cell receptor, there was an explosion of reagents to analyze the properties and diversities of the T-cell pool. One technical advance that allowed the discovery of CD8 clonal expansions was the development of monoclonal antibodies that recognized different TCR V alpha (Vα) and V beta (Vβ)

gene products. By using these reagents, investigators identified that young, healthy individuals had a relatively consistent number of T-cells expressing each Vα and Vβ gene product [18, 105]. In contrast, aging individuals frequently had significant perturbations in the abundance of T-cells expressing various Vα and Vβ gene products [18, 105]. Significantly, these aged individuals frequently had a massive overrepresentation of a single Vα or Vβ that was at least three standard deviations above the mean Vα and Vβ usage observed in young individuals. The selective outgrowth or accumulation of a single Vα or Vβ gene product within the CD8 T-cell pool suggested that these CD8 T-cells might be clonal expansions. Molecular analysis of the T-cell receptors used by these expanded populations of CD8 T-cells revealed that these overrepresented populations of CD8 T-cells were truly clonal [77, 105]. Notably, these clonal expansions were predominantly found within the CD8 T-cell lineage, and were rarely identified in CD4 T-cells.

Today, CD8 clonal expansions are frequently identified using antibodies against various Vβ gene products (Fig. 2). Based on this method, individuals with CD8 clonal expansions are identified as those with an overabundance of a single Vβ within the CD8 T-cell pool that is increased at least three standard deviations above the mean Vβ usage found for that Vβ in young individuals. The strength of this approach is that it identifies an overabundance of one Vβ within the entire CD8 T-cell pool. CD8 clonal expansions can also be identified by molecular analysis of TCR diversity (e.g. the spectratyping method [97]). When using such molecular methods, however, it is worth noting that these methods can detect reduced diversity within a specific Vβ gene family, despite the fact that that Vβ is not over-represented within the entire CD8 T-cell pool (a phenomenon we have referred to as clonal restriction [24]). Because of this caveat, we consider it preferable to identify the presence of clonal expansions by monoclonal antibodies against the TCR, followed by molecular analysis of TCR diversity. Age-associated clonal expansions are routinely clonal by such analyses.

One important observation about CD8 clonal expansions is that clonal expansions in different individuals express a diverse range of T-cell receptors. Even in genetically identical inbred mice that are housed together, CD8 clonal expansions express a wide variety of TCR Vαs and Vβs. The same is true for humans. Based on these observations, CD8 clonal expansions appear to arise from a diverse set of CD8 T-cells.

5 Properties of Clonal Expansions

5.1 Incidence and Abundance of Clonal Expansions in Humans and Mice

CD8 clonal expansions are a common age-associated alteration within the immune system. In specific-pathogen free (SPF) mice, almost 60% of mice develop clonal

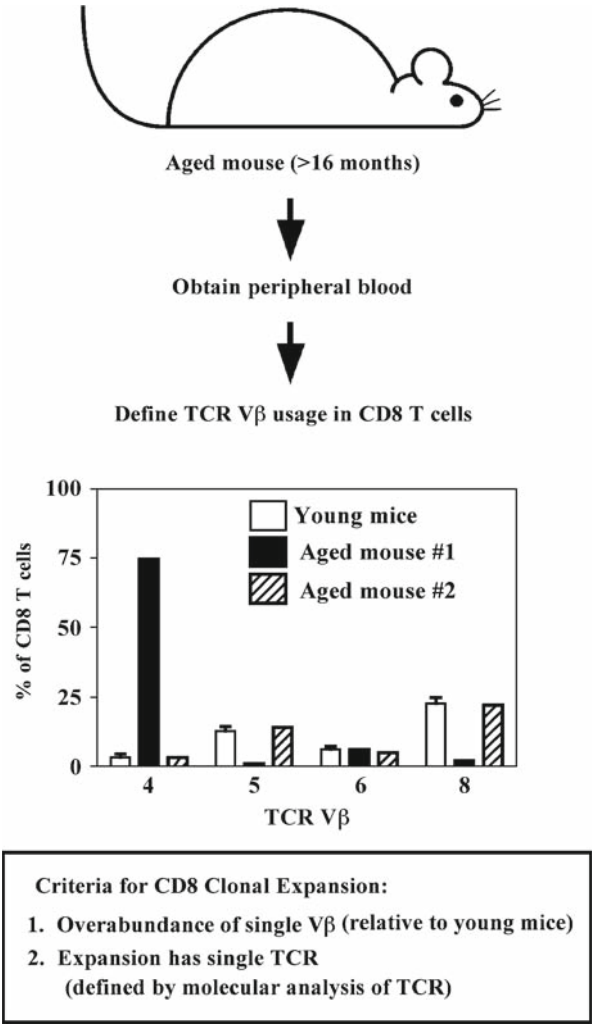

Fig. 2 Methodology to identify CD8 clonal expansions in aged mice
CD8 clonal expansions can be identified based on the percentage of CD8 T-cells expressing various TCR Vβ receptors. While young mice have a highly consistent percentage of CD8 T-cells that express each Vβ (open bars), certain aged mice (e.g., hypothetical aged mouse #1 in black bars) have an overabundance of CD8 T-cells expressing one Vβ (in this situation, a Vβ4+ clonal expansion). CD8 clonal expansions are identified in those mice that have an overabundance of one TCR Vβ, that is increased at least three standard deviations above the mean Vβ usage observed in a cohort of young mice. Data for young mice indicate mean Vβ usage +/- three standard deviations of the mean. Young mice are typically between 3–6 months of age. Aged mice develop detectable clonal expansions by 16 months of age. In this example, aged mouse #2 (hatched bar) does not have any detectable CD8 clonal expansions.

expansions by 2 years of age [71]. In humans, 33% of adults over the age of 65 have a detectable clonal expansion [108]. CD8 clonal expansions vary widely in their size within the CD8 T-cell pool. In the most dramatic situations, CD8 clonal

expansions can occupy 50% of the CD8 T-cell pool in humans [33] and 90% of the CD8 T-cell pool in mice (Clambey et al. unpublished data).

Since clonal expansions are found in many, but not all, mice, it is worth noting that studies of T-cell responses in aging mice may be profoundly influenced by whether an individual mouse contains a clonal expansion or does not. Given the idiosyncratic nature of clonal expansions, we strongly recommend that studies of T-cell function in aging mice be carefully controlled to minimize the impact of clonal expansions on the interpretation of the experiment.

5.2 Factors Associated with the Development of Clonal Expansions

While the precise origin of clonal expansions remains unclear, ongoing research has provided clues about potential cues that may facilitate the development of clonal expansions. One of the strongest factors associated with the development of clonal expansions is age. This age-association with clonal expansions is particularly pronounced in the mouse, where CD8 clonal expansions are virtually undetectable until 16 months of age [18]. In humans, increasing age is associated with an increasing prevalence of clonal expansions [108]. In contrast to mice, however, clonal expansions in humans can be found in younger individuals [108]. It is possible that these latter clonal expansions may reflect immune responses to childhood infections, something that would not be observed in SPF mice [50].

Although clonal expansions are particularly observed in aging individuals, it is unknown what factors within the aging environment, if any, contribute to the development of clones. It is worth noting that clonal expansions can be transferred to young individuals and still retain their competitive advantage [70, 91]. This observation indicates that while aging may contribute to the development of clonal expansions, the aging environment is not essential for the maintenance of clonal expansions.

Beyond the correlation of age and the development of clonal expansions, there are two other factors positively associated with the development of clonal expansions.

i) *Humans infected with human cytomegalovirus (HCMV)*. Over the past few years, the use of MHC Class I tetramers has allowed investigators to investigate the TCR specificity of human clonal expansions. Based on these studies, at least some human clonal expansions specifically recognize human cytomegalovirus, a common chronic herpesvirus infection [63, 98]. In the most dramatic case, 27% of CD8 T-cells in one individual were specific against a single HCMV epitope [98]. Although HCMV infection can be controlled in healthy individuals, there is increasing evidence that HCMV infection in the elderly is associated with a variety of negative outcomes [62, 102, 106, 122]. Importantly, while some humans develop clonal expansions against Epstein-Barr virus (EBV), another common chronic Herpes virus infection, these clonal expansions are

much smaller in size [62, 97, 129]. Thus, there appear to be certain factors associated with HCMV infection that are capable of eliciting pronounced CD8 clonal expansions in humans. This association may be related in part to the observation that chronic CMV infection can elicit large, and in some cases, highly focused T-cell responses that often increase in size with age, even in individuals without clonal expansions [57, 63, 127].

ii) *Lymphopenia and inflammation in mice*. Additional insights into cues that promote CD8 clonal expansions came from the analysis of CD8 clonal expansions in various mouse models. Significantly, mice characterized by lymphopenia (e.g., mice lacking the IL-7 receptor or mice subjected to adult thymectomy) develop clonal expansions at an earlier age and with a higher prevalence than intact, unmanipulated mice [90]. Although the precise mechanisms behind this outcome remain to be elucidated, one likely explanation for this effect is the increased rate of proliferation of CD8 memory T-cells in lymphopenic conditions [90]. In the same study, it was noted that repeated treatment of mice with adjuvants (compounds known to induce inflammation and to facilitate antigen-specific T- and B-cell responses to coinjected antigen) also modestly increased the incidence of CD8 clonal expansions [90]. It is interesting to note that states of inflammation, such as those of viral infections, have been associated with transient states of lymphopenia (e.g., [87]). Thus, it is possible that adjuvants promote the development of clonal expansions through the temporary generation of lymphopenic conditions. For further discussion of lymphopenia and its effects on the generation of clonal expansions please see chapter by Nikolich-Zugich.

5.3 CD8 Clonal Expansions may Impair Immune Function in the Aged

Given the dominance of CD8 clonal expansions within the aged individual, it is likely that clonal expansions have some impact on the immune function of aged individuals. To date, there are two studies to support this contention. First, in humans, there is a correlation between the presence of clonal expansions and an impaired response to influenza vaccination, a common defect in aged individuals [115]. Second, in mice, there are data that clonal expansions may result in highly focused holes in the T-cell repertoire (particularly in the Vβ subfamily used by the clonal expansion) [89]. These narrow holes may be particularly problematic in individuals responding to infections in which the T-cell response is heavily restricted to use of a single Vβ subfamily. Please see chapter by Nikolich-Zugich for more extensive discussion of the negative impact of clonal expansions on immune function in the aged.

While CD8 clonal expansions can have deleterious effects on immune function, we postulate that perhaps not all clonal expansions are deleterious to health

in the elderly. This may be particularly true in the case of clonal expansions specific for HCMV, a chronic virus infection that can cause disease, especially in the immune-suppressed. Although inflated responses to HCMV have often been viewed as a negative indication for health in the elderly (e.g., [102]), this is not to say that these HCMV-specific expansions are not playing some role in containing HCMV infection. Based on this concept, it will be important to test what consequence depletion of CD8 clonal expansions has in animal models of chronic infection (e.g., individuals which develop comparable clonal expansions in response to either mouse or primate cytomegalovirus infection) before further considering the possibility of therapeutic intervention to remove CD8 clonal expansions in the elderly.

One other important consideration when contemplating therapeutic interventions to remove CD8 clonal expansions in the aged is the effect that this depletion might have on subsequent T-cell homeostasis. For example, depletion of a clonal expansion that occupies 50% of the CD8 T-cell pool would likely create a transient state of lymphopenia, which may, in turn, provoke the subsequent development of another clonal expansion.

5.4 CD8 Clonal Expansions are Nonmalignant

Given the growth advantage of CD8 clonal expansions relative to other CD8 T-cells, one curious feature of clonal expansions is that they are nonmalignant. This conclusion is based on the following observations: i) individuals with CD8 clonal expansions do not have an increase in the total number of CD8 T-cells [89] and ii) clonal expansions can exist for an extended period of time without progressing to malignancy (up to 4 years in mice, up to 9 years in humans) (Ku, personal communication) [20]. Given the common occurrence of CD8 clonal expansions, the incidence of human tumors with a CD8 memory phenotype is extremely low [99]. It is important to note that individuals diagnosed with CD8 T-cell lymphomas not only have a clonal expansion of T-cells, but are also characterized by additional abnormalities (including elevated lymphocyte counts and frequent neutropenia) [110, 137]. At this time, there is no known relationship between those individuals with CD8 clonal expansions and those individuals who are diagnosed with T-cell lymphomas.

Despite the similarities of clonal expansions to tumors in terms of their clonality and competitive advantage relative to their neighbors, CD8 clonal expansions are clearly still subject to certain constraints. For example, CD8 clonal expansions do not increase in number above the normal number of CD8 T-cells contained within an individual [89]. Although the precise mechanisms that limit the growth of clonal expansions remain to be elucidated, we propose that a major factor constraining the growth of CD8 clonal expansions is availability for cytokines and other extracellular growth factors.

6 CD8 Clonal Expansions Have an Increased Rate of Proliferation

Many CD8 clonal expansions occupy a sizable fraction of the CD8 T-cell pool. The ability of expansions to out-compete other CD8 T-cells could result from either an increased rate of proliferation or from a decreased rate of attrition (e.g., apoptosis). Currently, there are no published reports rigorously examining the survival properties of CD8 clonal expansions relative to normal CD8 memory T-cells. In contrast, there are clear data regarding the rate of proliferation of CD8 clonal expansions. Initial evidence regarding the rate of proliferation of CD8 clonal expansions came from analysis of the rate of dilution of carboxyfluorescein diacetate succinimidyl ester (CFSE), a fluorescent dye that can be used to track the number of cell divisions of CD8 T-cells. Based on transfer of CD8 clonal expansions into syngeneic, nonirradiated recipients, CD8 clonal expansions had a modest increase in their rate of proliferation (dividing once every 15 days, compared with polyclonal aged CD8 memory T-cells which divided once every 22 days) [70]. Significantly, many CD8 clonal expansions were also capable of growing upon adoptive transfer into mice lacking beta-2 microglobulin and therefore having little to no MHC Class I ligands for the T-cell receptor [70]. This property is consistent with the previous observation that CD8 memory T-cells can achieve long-term antigen-independent proliferation [94]. In sum, these data indicate that clonal expansions have an increased rate of proliferation and that this proliferation is not dependent on active engagement between TCR and MHC.

In this initial study, manipulating cytokine signals also influenced the proliferation of CD8 clonal expansions. Clonal expansions had diminished proliferation when the beta-chain of the IL-2 and IL-15 receptors was blocked by antibody treatment, suggesting that clones were likely growing in response to IL-15 (a common proliferative cue for CD8 memory T-cells) [70]. In contrast, CD8 clonal expansions had accelerated proliferation when mice were treated with IL-2 antibodies [70], a condition now known to create a strong mitogenic signal for CD8 memory T-cells [13].

Since this initial analysis, an additional study examined how CD8 clonal expansions respond to conditions of lymphopenia, a condition known to increase the proliferative rate of CD8 T-cells and to promote the generation of LIP CD8 memory T-cells (discussed above). While these studies showed that CD8 clonal expansions have an increased rate of proliferation in a nonirradiated recipient relative to other CD8 T-cells, they also revealed a surprising finding: CD8 clonal expansions have a relatively constant rate of proliferation that is not accelerated in conditions of lymphopenia [91]. In this study, CD8 clonal expansions were also identified to have a modest increase in the expression of both the IL-7Rα and IL-2Rβ cytokine receptors [91]. Based on these studies, Nikolich-Zugich and colleagues proposed that CD8 clonal expansions have an altered capacity to respond to the homeostatic cues normally perceived by a CD8 T-cell [91]. On one hand, clones do not stop dividing in a full lymphoid compartment. On the other hand, clones do not accelerate their

division in a lymphopenic setting. At this time, it is unclear why clonal expansions are capable of accelerating their proliferation in response to strong mitogenic IL-2 signals [70], but do not accelerate their proliferation in lymphopenic settings [91]. One potential explanation for this apparent discrepancy may be that the proliferative cues perceived in a lymphopenic environment are less potent than that received by hyperstimulation with IL-2, IL-2 antibody complexes.

Based on the above data, clonal expansions do not simply have a higher rate of proliferation than other CD8 T-cells, but instead are capable of prolonged, continuous proliferation with little apparent regulation by the normal cues perceived by neighboring CD8 T-cells. One perplexing issue about these observations is that the state of lymphopenia is associated with an increased rate of development of clonal expansions, yet clonal expansions do not seem to have a proliferative advantage in the context of lymphopenia. One possible resolution for this paradox might be that lymphopenia promotes the initiation but not the maintenance of CD8 clonal expansions. For further discussion of this topic, please see chapter by Nikolich-Zugich.

7 The Spectrum of CD8 Clonal Expansions

7.1 Heterogeneous Characteristics of CD8 Clonal Expansions

One of the challenges in understanding CD8 clonal expansions in both mice and humans is the observation that distinct clonal expansions have variable properties. While heterogeneity between clonal expansions might be expected in humans, a genetically diverse population with significant differences in infection history, heterogeneity has also been observed between clonal expansions in genetically identical, inbred mice housed together [18]. To date, heterogeneity between clonal expansions has been best characterized in CD8 clonal expansions in mice [18], as described below.

i) *Stability of clones in vivo:* CD8 clonal expansions have widely discrepant stabilities in vivo. Some CD8 clonal expansions appear to be extremely stable and can continue to grow over a 4-year period, as revealed by serial adoptive transfer studies in mice (Ku, personal communication). In contrast, other CD8 clonal expansions are very unstable and disappear within 2 months of their initial identification [18, 77].
ii) *Response to stimulation in vitro:* CD8 clonal expansions in mice are also variable in their response to stimulation in vitro [18]. For example, some CD8 clonal expansions have a normal proliferative response to polyclonal stimulation in vitro (e.g., following culture with the concanavalin A or phorbol 12-myristate 13-acetate (PMA) and ionomycin). In contrast, other CD8 clonal expansions have an impaired capacity to proliferate and/or survive following similar stimulation conditions, becoming less abundant in bulk cultures following stimulation.

Despite these differences between CD8 clonal expansions, CD8 clonal expansions are uniformly considered to be CD8 memory T-cells, as defined by cell surface markers (in the mouse, $CD44^{high}$ as well as IL-7Rα^{high}IL-2Rβ^{high}) [70, 91]. Many CD8 clonal expansions belong to the T_{CM} subset of CD8 memory T-cells [91]. While our recent data (discussed below) have revealed additional heterogeneity in cell surface phenotypes, to date all clonal expansions are $CD44^{high}$, consistent with a CD8 memory phenotype in the mouse.

7.2 A New Method to Subclassify CD8 Clonal Expansions in Mice

Given the above heterogeneity between CD8 clonal expansions, we have been interested in identifying methods to subclassify clonal expansions in mice. To do this, we initially focused our efforts on microarray analysis in which we analyzed the transcriptional profile of multiple, independent clonal expansions in mice.

Through this analysis, we identified integrin α4 (also known as very late antigen-4 (VLA-4) or CD49d) as a candidate marker that was differentially expressed in distinct types of clonal expansions. [25] Next, we analyzed the expression of integrin α4 on a large number of age-associated CD8 clonal expansions. This analysis identified that there were two major types of clonal expansions: those expressing high levels of integrin α4 and those expressing low levels of integrin α4.

Based on the differential expression of integrin α4 between different types of clones, we analyzed the properties of integrin $\alpha4^{high}$ and integrin $\alpha4^{low}$ clones. There were clear differences between these two types of clonal expansions [25]. First, these clonal expansions were identified in mice of different ages, with integrin $\alpha4^{high}$ clones identified predominantly in mice 16–20 months of age, while integrin $\alpha4^{low}$ clones were found predominantly in mice 20–36 months of age. Notably, a longitudinal analysis of these two types of clonal expansions revealed that there was no interconversion between these integrin α4 phenotypes. Second, these clones differed in their in vivo growth dynamics, with integrin $\alpha4^{high}$ clones frequently decreasing in size over a 2-month interval, in contrast to integrin $\alpha4^{low}$ clones that rarely decreased in size. Third, integrin $\alpha4^{high}$ clones had an impaired response to in vitro stimulation with PMA and ionomycin, becoming less abundant following stimulation. Integrin $\alpha4^{low}$ clones had no advantage or disadvantage following this same stimulation. Fourth, integrin $\alpha4^{high}$ and integrin $\alpha4^{low}$ clones had differential localization in vivo. Integrin $\alpha4^{high}$ clones were absent from peripheral lymph nodes, while integrin $\alpha4^{low}$ clones were absent from Peyer's patches. Fifth, integrin $\alpha4^{high}$ clones had evidence of chronic TCR stimulation, as revealed by decreased expression of cytokine receptors (both IL-7Rα and IL-2Rβ) and expression of various inhibitory receptors (PD-1 and killer cell lectin-like receptor G1, KLRG1). In sum, integrin $\alpha4^{high}$ clonal expansions had many characteristics of chronic antigen stimulation, whereas integrin $\alpha4^{low}$ clonal expansions appeared similar to an antigen-independent CD8 memory T-cell [25].

The identification of integrin α4-defined clonal expansions in mice is significant for multiple reasons. First, it provides a molecular marker to distinguish between two types of clonal expansions with highly divergent properties. Second, it provides an explanation for the previous dichotomy observed in the properties of clonal expansions [18, 77]. Third, it indicates that these types of clonal expansions may have arisen from very different origins. In particular, we hypothesize that integrin $\alpha4^{high}$ clones may arise due to an inappropriate response against self-antigens, which would result in chronic antigenic stimulation. It is worth noting that while both types of clonal expansions meet the current definition of CD8 clonal expansions, integrin $\alpha4^{low}$ clones appear to be the subtype of expansion that is most capable of long-term growth.

8 Models Regarding the Development and Properties of CD8 Clonal Expansions

8.1 Models to Understand the Development and Properties of Clonal Expansions

Given the heterogeneity between distinct clonal expansions and the apparent differences in clonal expansions between mice and humans (discussed in further detail in [24]), it is challenging to determine whether there are common mechanisms underlying divergent types of CD8 clonal expansions. Here we discuss three conceptual models for the development of CD8 clonal expansions and discuss basic tenants of each model.

Model 1: Clonal expansions arise from natural variation in the rate of proliferation of memory T-cells. Clonal expansions are simply those memory T-cells with the fastest rate of proliferation.

Basic details of this model: This model is based on the principle that there is a range of proliferative rates of memory T-cells present in a normal individual. While the vast majority of cells will proliferate at a very similar rate, there inevitably will be some cells that proliferate slightly faster or slower. At first inspection, this idea is particularly appealing: CD8 clonal expansions only have a modest increase in their rate of proliferation (dividing about once every 15 days compared to CD8 MP T-cells which divide about once every 22 days) [70].

Predictions of this model: CD8 clonal expansions will be identical to CD8 memory T-cells in all parameters, with only a modest acceleration in their rate of proliferation.

Evidence against this model: The major observation that is inconsistent with this model is that CD8 clonal expansions have an altered capacity to respond to proliferative cues typically perceived by lymphocytes in a lymphopenic environment [88]. This property of clones is clearly different than a normal CD8 memory T-cell, and these data indicate that clones are not simply derived from the fastest cell in the

CD8 memory T-cell pool. Despite this, it is worth noting that subtle variations in the expression level of cytokines receptors or inhibitory proteins may still play some role in the basic biology of CD8 clonal expansions.

Model 2: Clonal expansions arise from common alterations to growth regulatory pathways. The variable properties of clonal expansions reflect differences in TCR reactivity and antigen persistence.

Basic details of this model: This model proposes that clonal expansions arise from a discrete set of changes in the expression of growth regulatory proteins (e.g. cell cycle inhibitory proteins or cytokine receptors) (Fig. 3a). These alterations in mRNA or protein expression and/or function may arise to due genetic mutations (i.e., creating mutant gene products) or due to perturbations in epigenetic regulation (e.g., DNA methylation or chromatin alterations that alter transcriptional expression of growth regulatory genes).

Predictions of this model: A basic prediction of this model is that clonal expansions will arise from a common fate, and possess common changes in growth regulatory pathways (e.g., cytokine signaling). Moreover, clonal expansions with divergent biological properties should have similar mRNA and protein expression profiles (discussed further below).

Evidence against this model: Currently, there are two pieces of evidence against this model. The first is that the two major types of clonal expansions in mice (integrin $\alpha4^{high}$ and integrin $\alpha4^{low}$) have widely divergent properties, suggesting that they may arise from different age-associated alterations. Integrin $\alpha4^{high}$ clones have many characteristics consistent with T-cells actively responding against chronic (potentially self) antigen. If these cells are self-reactive, integrin $\alpha4^{high}$ clones may arise from age-associated alterations in central or peripheral T-cell tolerance. In contrast, integrin $\alpha4^{low}$ clones do not possess such characteristics, suggesting that they may arise from a distinct mechanism (such as epigenetic inactivation of a growth regulatory gene). The second piece of evidence against this model is microarray analysis, in which integrin $\alpha4^{high}$ and integrin $\alpha4^{low}$ clonal expansions appear to have different gene expression profiles (Clambey et al. manuscript in submission). The interpretation of this latter point, however, has caveats (discussed below), and will require analysis of a larger number of integrin α4-defined clonal expansions.

Model 3 : Clonal expansions arise from multiple, distinct age-associated alterations.

Basic details of this model: In contrast to model 2 (above), this model proposes that clonal expansions reflect a common physiological outcome (i.e. selective outgrowth of a single CD8 T-cell), but that these clones arise due to different age-associated alterations (Fig. 3b). As such, different types of clonal expansions have little in common other than their overabundance in the CD8 T-cell pool.

Predictions of this model: In contrast to model 2, a prediction of this model is that different types of CD8 clonal expansions (with different biological properties) will have different gene and protein expression profiles. While different types of clones will have some common expression profiles since they are both CD8 memory T-cells, the underlying molecular changes and growth requirements for these clones will differ. Based on the properties of integrin $\alpha4^{high}$ and integrin $\alpha4^{low}$ clones detailed above, integrin $\alpha4^{high}$ clones would depend on ongoing antigen/

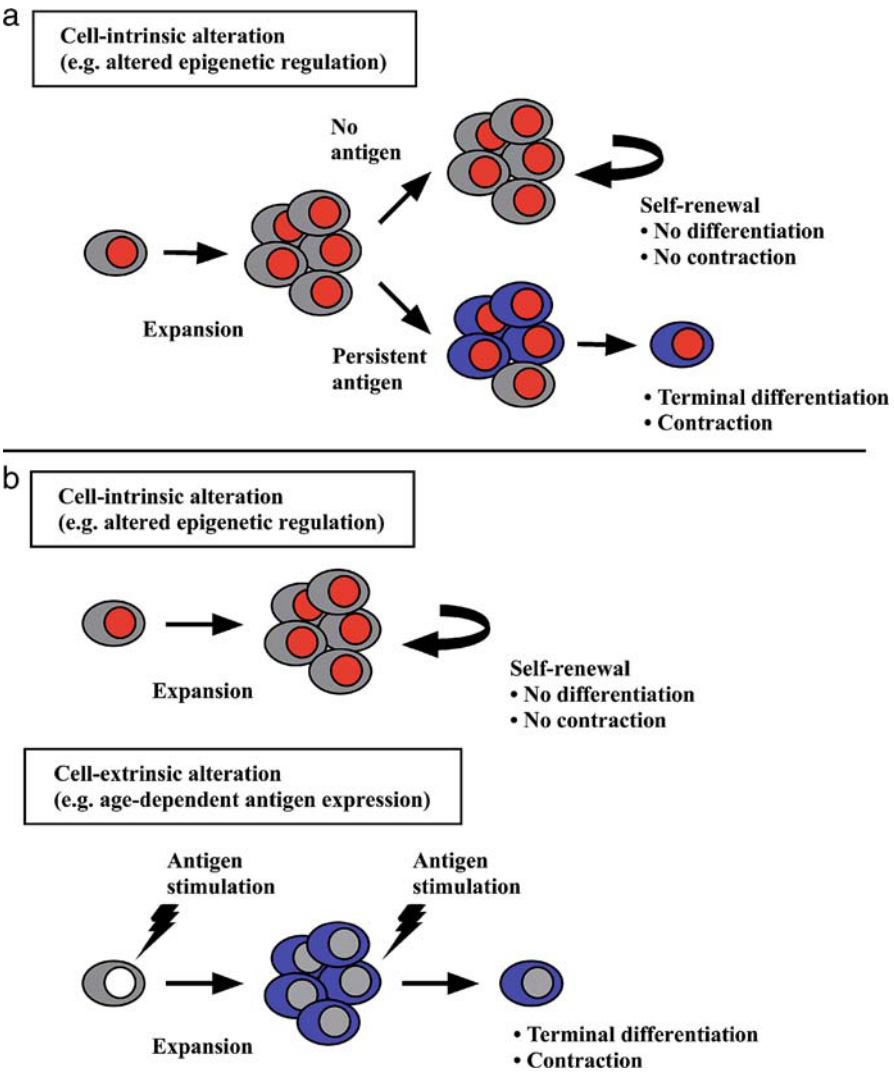

Fig. 3 Models for the development and phenotype of CD8 clonal expansions
In panel a, model 2 (see text for details) proposes that CD8 clonal expansions result from a common set of changes in growth regulatory genes (indicated here by a red nucleus, denoting a common transcriptional alteration). Following their initial expansion, the presence or absence of antigen then significantly influences the properties and dynamics of the clonal expansion. Clones that recognize persistent antigen undergo further differentiation (indicated by a blue cytoplasm). In panel b, model 3 (see text for details) proposes that CD8 clonal expansions result from distinct age-associated changes. While these distinct changes both result in a CD8 clonal expansion, the underlying factors that promote these expansions are completely distinct (indicated by either a red nucleus representing a transcriptional alteration or a blue cytoplasm representing antigen driven stimulation). See text for further discussion of each model.

TCR engagement for their proliferative advantage, whereas integrin $\alpha 4^{low}$ clones would not.

Evidence against this model: Currently there is no direct evidence that contradicts this model.

One important limitation to the above models and predictions is our current inability to distinguish between changes in gene expression profiles that promote the growth of CD8 clonal expansions, compared to changes in gene expression profiles that reflect TCR specificity and the presence or absence of antigen. For example, a clonal expansion responding to a persistent antigen will have major alterations in gene expression (e.g., in the expression of inhibitory receptors such as PD-1). As such, it may be very difficult to discriminate between the influence of TCR and antigen versus the underlying mechanism that creates a clonal expansion. The ability to resolve these issues will only become possible when CD8 clonal expansions can be reliably generated with defined antigen specificities, and such clones can be analyzed in the context of varying conditions of antigen persistence. Future insights into the molecular bases of clonal expansions will be significantly advanced through gain- and loss-of-function studies in both CD8 clonal expansions and CD8 memory T-cells.

8.2 The Probability of Becoming a Clonal Expansion

With regard to models 2 and 3, both models predict that certain stochastic events would change the growth properties of a CD8 memory T-cell. We postulate that this growth-promoting event is a relatively rare event. This statement is based on the observation that not all mice appear to develop CD8 clonal expansions, and mice that do develop clonal expansions frequently only have one clone (Clambey et al.unpublished data). Given that each mouse has more than 1 x 10^7 CD8 T-cells, the frequency of this growth-promoting event in an aging immune-competent mouse (e.g., C57BL/6J mouse) is probably not more frequent than 1 in 10^7 cells. We predict that the likelihood that a particular CD8 T-cell specificity becomes a clonal expansion would be influenced by the overall abundance of that antigen specificity within the CD8 T-cell pool (further discussed in [24]).

It is interesting to note that lymphopenic mice, which have fewer CD8 T-cells, have an accelerated rate of clonal expansion development, as well as a higher overall incidence of clones [90]. Based on these data, the frequency of the growth-promoting event is increased in conditions of lymphopenia. Since lymphopenic mice are characterized by a higher number of proliferating CD8 T-cells [90], we hypothesize that the probability that a growth-promoting event occurs is directly related to the number of cell divisions that the CD8 T-cell has undergone. Mechanistically, this hypothesis is based on the fact that with each cell division, appropriate epigenetic programming must be perpetuated from the mother to the daughter cells. If there is a certain rate of failure for this event to occur, the more rounds of cell division, the more likely it is that any cell would undergo this growth-promoting event.

8.3 When Do Clonal Expansions Initially Emerge?

In mice, CD8 clonal expansions are not detected until mice are approximately 16 months of age [18]. Although clonal expansions become apparent at this age, it is likely that there is a period during which an emerging clonal expansion remains below the limit of detection within the T-cell repertoire. The events within the early phase of clonal expansions are completely unknown, and at this point, strictly hypothetical. Nonetheless, it is worth considering how long it might take for a clonal expansion to emerge and dominate the CD8 T-cell pool.

CD8 clonal expansions divide once every 15 days, in contrast to polyclonal CD8 MP T-cells that divide once every 22 days [70]. If this was the only advantage that a CD8 clonal expansion had relative to other CD8 T-cells, how long would it take for a clonal expansion to outcompete its neighbors?

In an attempt to approximate the growth history of a clonal expansion, we have used a very simple model to compare the growth dynamics of a clonal expansion relative to a pool of CD8 MP T-cells. In this model, we made the following assumptions:

i) a clonal expansion results from a single CD8 memory T-cell achieving a growth advantage relative to its neighbor
ii) the only advantage that a clonal expansion has relative to other CD8 memory T-cells is its slightly higher rate of proliferation (dividing once every 15 days, instead of once every 22 days)
iii) the relative size of the proliferating, polyclonal CD8 MP T-cell pool contains approximately 10 x 10^6 cells (Clambey et al. unpublished data) [11, 47, 89] and
iv) both the clonal expansion and the CD8 MP pool have an infinite growth capacity.

While this model is clearly too simplistic (e.g. it does not take into consideration rate of death nor the changing abundance of naïve T-cells with age), it does provide a very useful piece of information (Fig. 4). If a clone only has this subtle growth advantage relative to a large CD8 MP T-cell pool, it would take 855 days (approximately 28.5 months) for the clone to reach just 5% (a small clonal expansion) of the size of the CD8 MP T-cell pool (Fig. 4). However, in mice, larger CD8 clonal expansions are already detectable by 16 months of age (~480 days). Based on this, clonal expansions are likely to have additional factors which promote their dominance within the CD8 T-cell pool.

One condition that could expedite the dominance of a clonal expansion within the CD8 T-cell pool is if there were a higher starting number of cells with a growth advantage. Since clonal expansions are clonal, however, cells with a growth advantage would need to come from a common precursor. One way in which this could happen is if a naïve T-cell achieves a growth-promoting event, and then encounters its antigen. Notably, this growth-promoting event does not need to change the antigen driven proliferation phase or the extent of death following the

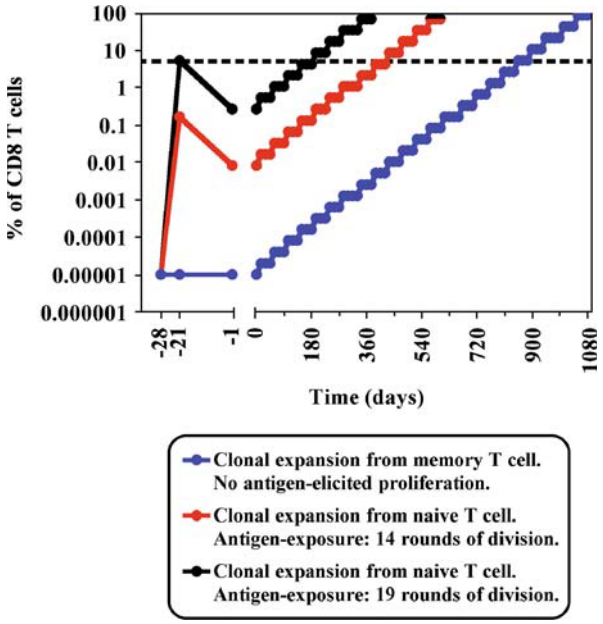

Fig. 4 A simple model to predict how long it takes for a clonal expansion to achieve dominance within the CD8 T-cell pool

Graph indicates the relative abundance of a clonal expansion (dividing once every 15 days) relative to the size of a pool of ten million proliferating CD8 MP T-cells (dividing once every 22 days). This model compares three different growth projections for an emerging clonal expansion: i) in blue, a single CD8 memory T-cell achieves a growth-promoting event that results in an increased rate of proliferation (expansion divides once every 15 days, compared to CD8 MP T-cells that divide once every 22 days) or ii) in black and in red, a single naïve T-cell achieves a growth-promoting event, followed by antigen stimulation. After antigen stimulation, the naïve T-cell goes through a normal phase of expansion and contraction. In contrast to the normal CD8 memory T-cell pool, however, the resulting CD8 memory T-cells all contain a common growth–promoting event that confers an increased rate of proliferation (clonal expansion divides once every 15 days, compared to CD8 MP T-cells that divide once every 22 days). For this latter model, growth projections include two different estimates for the extent of naïve T-cell proliferation following antigen stimulation (either 14 rounds of division indicated in red, or 19 rounds of division indicated in black). Antigen driven proliferation and contraction (95% of cells dying by apoptosis) are indicated from day—28 to day—1. Dashed line indicates 5% of the CD8 T-cell pool, which is a conservative estimate for the detection of a CD8 clonal expansion. Each data point indicates the relative abundance of the clonal expansion relative to the CD8 MP T-cell pool with each round of division (occurring every 15 days). This model presumes that both the clonal expansion and the CD8 MP T-cell pool have an infinite growth capacity, and does not take into consideration rate of death for either population (this parameter is undefined for clonal expansions at this time). See text for further details of model.

peak of the response. Instead, this growth-promoting event simply needs to increase the basal rate of proliferation of the resulting CD8 memory T-cells (so that the resulting cells divide once every 15 days). The end-result of this outcome would be that there would be a higher number of memory cells with a growth advantage. This, in turn, dramatically alters the time required for the clonal expansion to domi-

nate the CD8 T-cell pool. By using a conservative estimate for how many times a naïve T-cell proliferates following an acute antigen exposure (14 rounds of division [3, 10, 84]), a clonal expansion can achieve 5% of the CD8 T-cell pool within 435 days (14.5 months), and occupy more than 30% of the repertoire within 535 days (17.5 months) (Fig. 4, red line). If a naïve T-cell undergoes 19 rounds of division (a recent estimate for naïve T-cell proliferation during acute infection [3]), it would only take 195 days (6.5 months) to achieve 5% of the CD8 T-cell pool, coming to occupy >30% of the repertoire within 285 days (9.5 months).

The bottom line from this overly simplistic model is that although a subtle increase in proliferation may contribute to the development of clonal expansions, there are likely to be other contributing factors. For example, changes in the rate of death could significantly influence the ability of clones to compete with other CD8 T-cells; in addition, if a clone ever goes through a proliferative burst (e.g., following antigen engagement) this could also accelerate the development of clonal expansions. Future reductionist studies will allow a more careful dissection of the time required for a CD8 T-cell to become a clonal expansion.

9 Factors that Influence the Properties of CD8 Clonal Expansions

Regardless of the precise mechanisms that are behind the development of clonal expansions, the phenotype and properties of individual clones are certain to be influenced by multiple factors, most importantly the interaction between the TCR and antigen.

9.1 The Role of Antigen Persistence

It is increasingly clear that the persistence of antigen significantly impacts the phenotype and dynamics of the CD8 T-cell. While CD8 T-cells only require a very brief period of antigen engagement of the T-cell receptor to become activated [53, 128, 139], the duration and context of antigen presentation can significantly influence the capabilities of the resulting CD8 T-cell. The crippling effects of chronic antigen exposure can be best observed in certain models of chronic infection, where CD8 T-cells never differentiate to a state in which they can survive in the absence of antigen [120, 131].

With regard to CD8 clonal expansions, clonal expansions encountering chronic antigen would be predicted to have significantly different cell surface phenotypes (Fig. 3). These changes in cell surface phenotype would likely influence the expression of cytokine receptors for IL-7 and IL-15 (possibly influencing IL-7Rα and IL-2Rβ), as well as result in the upregulation of various inhibitory receptors (such as PD-1 and KLRG1, which are both receptors whose expression is associated

with chronically stimulated T-cells [6, 126]). In addition, these clonal expansions would be predicted to disappear if antigen ultimately disappears.

Although chronic antigen exposure would most significantly impact the phenotype and properties of clonal expansions, initial encounter of antigen could also play a more modest effect on the resulting phenotype, for example influencing the fate of the resulting CD8 memory T-cell to become a T_{CM} or a T_{EM} cell.

9.2 The Impact of Initial Conditions of Stimulation

As discussed above, a naïve CD8 T-cell can differentiate into a CD8 memory T-cell by at least two distinct paths: i) engagement of the TCR by its appropriate antigen, resulting in the full activation of the T-cell, followed by subsequent proliferation, contraction and differentiation (referred to as antigen-elicited memory) or ii) lymphopenic conditions in which a naïve CD8 T-cell is capable of undergoing proliferation in the absence of full activation (referred to as LIP memory). While the precise characteristics of these two types of memory cells is a subject of ongoing investigation, it is worth noting that there are surprisingly few differences in the properties of these two types of memory cells. Careful microarray analysis of these two types of memory cells has revealed very similar transcriptional profiles [36], and LIP memory cells can mediate immunological protection, a hallmark of memory T-cells [39]. Although no obvious differences between these two types of memory cells have been identified to date, the very different conditions from which they originate make it highly unlikely that they are absolutely identical.

With regard to CD8 clonal expansions, it appears that CD8 clonal expansions may become CD8 memory T-cells through either an antigen-elicited or LIP mechanism. This conclusion is based on the following data:

i) in mice, conditions of lymphopenia are associated with the accelerated development of clonal expansions indicating that LIP memory cells can become CD8 clonal expansions [90]
ii) mice infected with certain infections such as Sendaivirus, influenza, Herpes simplex virus or LCMV can occasionally develop very large antigen-specific clonal expansions in aged mice ([29, 74], Zajac, personal communication), indicating that antigen-elicited memory cells can become CD8 clonal expansions (see chapters by Woodland and Nikolich-Zugich for further discussion),
iii) humans infected with HCMV can develop HCMV-specific clonal expansions [63, 98].

Given that both LIP and antigen-elicited memory CD8 T-cells can become CD8 clonal expansions, it is worth noting that the relative contribution of these two types of memory cells differs between SPF mice and humans. For example, in SPF mice that are typically used to study clonal expansions in mice, the majority of clonal expansions almost certainly represent LIP memory T-cells given the relative paucity of antigen exposure these animals experience. In contrast, humans have an extremely high rate of

antigen exposure, with relatively few memory CD8 T-cells likely to arise from lymphopenia-induced proliferation. Based on this difference, we postulate that the majority of human clonal expansions will recognize a variety of antigens primarily from infectious agents, whereas the majority of clonal expansions in mice will recognize a wide variety of antigens without a bias for infectious agent antigens. Despite this difference, exposure of mice to a variety of infections should be capable of recapitulating the diversity of antigen-elicited clonal expansions that we postulate to occur in humans.

10 Major Questions about CD8 Clonal Expansions

CD8 clonal expansions are a common age-associated perturbation in the immune system. The goal in studying this phenomenon is that it will reveal previously unappreciated effects of the aging environment on CD8 memory T-cell homeostasis, and identify basic cellular and molecular factors that also regulate CD8 memory T-cells in healthy, young individuals. While there is increasing information about this phenomenon, there are still many unanswered questions:

1. How does the aging environment influence the development of CD8 clonal expansions?
2. What are the molecular alterations that contribute to increased growth of CD8 clonal expansions?
3. What factors constrain the growth of CD8 clonal expansions?
4. What is the underlying cause for the heterogeneous phenotype of CD8 clonal expansions?
5. Can every subset of CD8 memory T-cell become a clonal expansion?

We anticipate that research in the upcoming years will shed light on many of these questions, providing new insights into how the aging immune system influences the dynamics of CD8 memory T-cell homeostasis.

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Generation and Gene Expression of CD28$^-$ CD8 T-cell in Human

Nan-ping Weng

Contents

Abstract: Increase of CD28$^-$CD8 T-cells is one of the hallmarks of aging in the human immune system. Recent studies reveal the mechanism of generation and gene expression features of CD28$^-$CD8 T-cells. Here, I summarize the recent progress focusing on the role of interleukin-15 (IL-15) in generation of CD28$^-$CD8 T-cells and the identification of unique gene expression in CD28$^-$CD8 T-cells by microarray gene expression analysis. These new findings enhance our understanding of the origin and function of the CD28$^-$CD8 T-cells and may provide new means for clinical intervention.

1 Overview

CD8 T-cells play an essential role in the control of intracellular pathogens and cancerous growths for the host. The capability of the immune system, particularly CD8 T-cells, to protect the host declines with age [1, 2]. Accumulating evidence suggest that increase of CD28$^-$CD8 T-cells in peripheral blood, a consistent age-associated change, account for the decline of CD8 T-cell mediated protection in the elderly [3–6]. However, the mechanisms underlying the age-associated changes in the immune system are complex and have just begun to be understood.

CD28, a membrane glycoprotein serving as a major co-stimulatory receptor for TCR mediated activation, plays multiple roles during T-cell activation from amplification of the TCR signal to induction of key cytokine production such as IL-2 to

N.-p. Weng (✉)
Laboratory of Immunology
National Institute on Aging, National Institutes of Health
251 Bayview Blvd, Suite 100, Baltimore, Maryland 21224
E-mail: wengn@mail.nih.gov

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ensure a complete activation of T-cells after stimulation with antigen [7, 8]. Loss of CD28 expression has profound impact on the function of T-cells [9]. For example: (1) decreased production of IL-2 and IFN-γ in response to stimulation [10]; (2) resistance to apoptosis [11]; (3) reduced antigen repertoire diversity [5], and (4) associated with the lack of antibody production after immunization [12]. In addition, $CD28^-$CD8 T-cells gain expression of some NK cell markers such as KIRs, CD16, CD56, KLRK1 (NKG2D), and retain or increase cytotoxicity with high expression of granzyme B and perforin [3].

Despite recent findings, the key issues related to $CD28^-$CD8 T-cells remain to be elucidated. What are the causes of $CD28^-$CD8 memory T-cells? How $CD28^-$CD8 memory T-cells are maintained in vivo? What are the molecular features of $CD28^-$CD8 memory T-cells compared to $CD28^+$CD8 memory T-cells? In this chapter, I review the ontogeny of $CD28^-$CD8 memory T-cells and summarize the genome-wide analysis of gene expression profiles of $CD28^-$CD8 memory T-cells from peripheral blood. I will discuss the features of gene expression of $CD28^-$CD8 memory T-cells as compared to their $CD28^+$ counterparts.

2 Ontogeny of $CD28^-$CD8 T-cells

In newborn human, all T-cells in the peripheral blood express CD28 on the cell surface [3, 13]. As $CD28^-$ T-cells appear after birth and gradually increase with age [14], it has been suggested that $CD28^-$CD8 T-cells are derived from $CD28^+$CD8 T-cells. In the past decades, accumulating evidence support such a notion and the causes of $CD28^-$CD8 T-cells are begun to be understood. There is overwhelming evidence indicating that repeated antigenic stimulation, mostly viral challenge, is one major cause of down-regulation of CD28 expression in T-cells [15, 16]. More recently, homeostatic cytokines such as IL-15 are also capable of induce down-regulation of CD28 expression in CD8 T-cells [17, 18].

2.1 Antigenic Stimulation Induced $CD28^-$CD8 T-cells

An increasing number of publications shows that increase of $CD28^-$CD8 T-cells are found in patients with a variety of viral infections including human immunodeficiency virus (HIV) [19, 20], cytomegalovirus (CMV) [21, 22], Epstein-Barr virus (EBV) [23, 24], and Hepatitis C virus (HCV) [25, 26]. A common feature of these viral infections is relative persistent in the host and their interaction with immune system is often long lasting and results in varing degree of increase of effector T-cells, particularly in the CD8 T-cells. In the CD8 T-cell compartment, most of these responding CD8 T-cells are $CD28^-$CD8 T-cells. The notion that these $CD28^-$CD8 T-cells are derived from their precursor $CD28^+$CD8 T-cells after viral

stimulation is supported by several findings. (1) CD28$^+$CD8 T-cells stimulated in vitro loss CD28 expression to become CD28$^-$CD8 T-cells [4]. The loss of CD28 expression in these viral antigen primed CD8 T-cells appears stable, which is different from a transient down-regulation of CD28 expression on T-cells occurs after antigenic stimulation [27].

2.2 Cytokine Mediated Loss of CD28 Expression in CD8 T-cells

Recently, down-regulation of CD28 expression in T-cells by cytokines sharing the common γ-chain receptors has been reported [17, 18]. Although Borthwick showed that IL-2, IL-7, and IL-15 were capable of down-regulation of CD28 expression in T-cells after a short term culture, it is unclear if such down-regulation is transient or stable and what mechanisms are responsible for these cytokine-mediated down-regulations of CD28. In addition, TNF-α, a proinflammatory cytokine secreted by various types of cells including T-cells, has also been shown to down-regulate CD28 expression in CD4 T-cells [28]. However, the relationship of cytokines of the common γ-chain family and the TNF family in down-regulation of CD28 expression is not fully understood.

Loss of CD28 expression in memory CD8 T-cells under homeostatic cytokine IL-15 in a longer term of culture has been analyzed in more detail [18]. In general, CD28 expression was relatively stable during the initial few rounds of cell divisions under IL-15 but a significant loss of CD28 expression occurred after the fifth cell division. The average ratio of CD28$^-$ to CD28$^+$CD8 memory T-cells is 0.43 for the cells that had undergone fewer than five cell divisions while this CD28$^-$ to CD28$^+$CD8 memory T-cell ratio increases to 1.4 in cells undergone five or more cell divisions. Further analysis to determine if loss of CD28 expression was limited to the surface expression or occurred at the transcription level, we found that CD28 mRNA was absent in CD28$^-$CD8 memory T-cells, suggesting that the down-regulation of CD28 expression under IL-15 is at transcriptional level. Finally, the loss of CD28 expression in CD8 memory T-cells is quite stable under IL-15-culture and there is no obvious re-gain of CD28 expression in those CD28$^-$CD8 T-cells over a month of culture. These findings suggest that IL-15-mediated down-regulation of CD28 expression occurs primarily in actively dividing CD28$^+$CD8 memory T-cells and that IL-15-induced loss of CD28 expression in CD8 memory T-cells is stable under continuous IL-15 stimulation.

How does IL-15 induce down-regulation of CD28 expression in CD8 memory T-cells? We found that IL-15 induced production of TNF-α in CD8 memory T-cells and blocking TNF-α effect with the neutralizing anti-TNF-α antibody reduced CD28$^-$CD8 T-cells by approximately 15% (p=0.002, n=12) after 14-day IL-15 culture [18]. More dramatically, supplement of recombinant TNF-α(200 ng/ml) in IL-15 culture induced significantly more CD28$^-$CD8 T-cells than that of the control cultures (IL-15 alone) at day 14 (195% increase, p= 7.1×10^{-6}). The loss of CD28

expression in CD8 memory T-cells induced by exogenous TNF-α is time and dosage-dependent. Supplement of recombinant TNF-α at the beginning of culture accelerated CD28 down-regulation in CD8 memory T-cells as early as 7 days of culture. The effect of TNF-α on down-regulation of CD28 expression in IL-15 cultured CD8 memory T-cells is seen as low as 50 ng/ml of TNF-α. Together, these findings indicate that IL-15 induced down-regulation of CD28 expression in CD8 memory T-cells is partially through production of TNF-α.

2.3 Transcriptional Regulation of CD28 Expression in CD8 T-cells

Loss of CD28 expression in $CD28^-$CD8 T-cells appears to be regulated at the transcription level [29, 30]. Analysis of the promoter of CD28 reveals that an inoperative transcriptional initiator (INR) consisting of two motifs α and β at the proximal region of CD28 promoter is involved in the regulation of transcription of CD28 [30]. Loss of α and β bound complexes is found in $CD28^-$ T-cells and two proteins, nucleolin and heterogeneous nuclear ribonucleoprotein-D0 isoform A (hnRNP-D0A), bind to the α motif of INR [31]. The binding of these proteins to the α motif of INR is required for transcription of CD28 as lack of nucleolin and hnRNP-D0A at the α site INR site appears to be associated with the loss of transcription of CD28 in $CD28^-$ T-cell lines [31]. Because these findings were derived from cell lines, it remains to be confirmed if the same regulation works in normal/primary T-cells during chronic infection and replicative senescence. Equally important is to understand how the chronic stimulation and/or replicative senescence lead to the loss of the α/β INR complexes in the promoter of CD28 gene.

3 Gene Expression Analysis of $CD28^-$CD8 T-cells

3.1 Experimental Design

Memory phenotype CD8 T-cells that are $CD28^+$ and $CD28^-$ were isolated from peripheral blood of healthy adults based on the surface markers CD8, $CD45RA^-$ and CD28 by cell sorting. The purity of sorted $CD28^+$ and $CD28^-$ memory phenotype CD8 T-cells was over 95%. Total RNA were extracted immediately from half of the sorted cells or after 5-day culture with human recombinant IL-15 (50 ng/ml, Peprotech, Boston, MA) of the rest of sorted cells. The quality and quantity of total RNA were analyzed by an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) and only the high quality RNA were used in the microarray experiment and in real time quantitative RT-PCR. To minimize the potential differences among individuals, RNA was pooled from 2–3 donors and a total of three RNA pools were generated for microarray experi-

ments and for real time quantitative RT-PCR analysis. A fourth RNA pool was made for additional real time quantitative RT-PCR analysis to ensure the changes identified here were common between $CD28^+$ and $CD28^-$CD8 memory T-cells.

The microarray gene chips were purchased from Agilent Technologies (Whole Human Genome Oligo Chip). This Whole Human Genome Oligo Chip consists of the vast majority of the genes and transcripts in human genome (36,866) on a single slide. The targets on the chip were 60-mer oligonucleotides which offer an overall excellent balance between sensitivity and specificity. The two-fluorescent dyes detection system with a standard universal reference RNA was used in the signal detection to allow a uniformed comparison among different chips. As only three biological replications in this experiment, we applied a conservative error model to reduce the false positives. Statistical significance was determined using the false discovery rate (FDR). The FDR was set to 0.05, which corresponds to the average proportion of false positives = 5% in combination with the pair-wise mean comparison of the signal intensity difference was set to be greater than 2 fold. Finally, real time RT-PCR was applied to independently confirm these selected significant genes. Most of them were confirmed by real time RT-PCR, and the agreeable rate was 85%.

3.2 Gene Expression Changes in CD28⁻CD8 Memory T-cells

Overall, $CD28^-$ and $CD28^+$CD8 memory T-cells expressed similar number of genes at the comparable levels. A small number of genes (58 out of 36,866 analyzed) displayed significant difference in mRNA level between $CD28^-$ and $CD28^+$CD8 memory T-cells. The majority of these differentially expressed genes are known genes (78%, 45 out of 58 genes) and they serve a wide range of functions and are discussed below.

3.2.1 Expression of Co-Stimulatory Receptors in CD28⁻CD8 T-cells

The CD28 co-stimulatory receptor family consists of five known members, CD28, CTLA-4 (CD152), inducible costimulator (ICOS), program death-1 (PD-1), and B and T Lymphocyte Attenuator (BTLA) [8]. The CD28 family transmembrane proteins have a single extracellular IgV domain and a cytoplasmic tail. CD28 is constitutively expressed on the cell surface of most T-cells and plays a primary role in augmenting TCR signals upon activation. The expression of CD28 decreases after activation. CTLA-4 increases expression after activation and serves as an inhibitory receptor [32, 33]. ICOS expression increases after activation and may play a role in sustained stimulation of effector functions of T-cells [34]. PD-1 and BTLA are both expressed on T and B cells and serve as inhibitory receptors [35, 36]. Among the five members of CD28 family, we found that only CD28 expression was significantly diminished in $CD28^-$CD8 memory T-cells compared to $CD28^+$ counterparts.

CD40L and CD70 are members of the TNF superfamily and both serve as co-stimulatory receptors during T-cell activation. The expression pattern of CD40L is similar to CTLA4 during CD8 T-cell differentiation. CD70 is a ligand for CD27, a receptor that is member of the tumor necrosis factor receptor (TNFR family). The signal generated from CD27/CD70 interaction is temporally or spatially segregated from CD28 during T-cell activation [37]. The T-cell activation/survival signals generated by different co-stimulators have some functions in common and yet distinct from each other in other aspects to allow effectiveness and longevity of the T-cell response and survival. The levels of CD27 and CD70 are stable from naive to memory ($CD28^+$ to $CD28^-$) cells. Decreased CD27 expression associated with increased CD70 expression are found in the effector memory CD8 T-cells. The significance of this altered balance of CD27/CD70 expression remains to be determined. It is clear, however, that the parallel loss of CD27 and CD28 expression has profound impact on CD8 T-cell function.

Not all co-stimulatory receptors were down-regulated in $CD28^-$CD8 memory T-cells. Two co-stimulatory receptors (4-1BB and SLAMF7) express higher in $CD28^-$CD8 memory T-cells. 4-1BB (CD137) belongs to the TNFR gene family and plays a key role in activation-induced cell division, survival, and effector function of CD8 T-cells [38, 39]. An increased 4-1BB expression along with a diminished expression of CD154 coexists in $CD28^-$CD8 memory T-cells, suggesting that an elevated 4-1BB could facilitate the growth and survival of $CD28^-$CD8 memory T-cells in vivo. If this is true, 4-1BB might facilitate the age-associated accumulation of $CD28^-$CD8 T-cells. CD2-like receptor activating cytotoxic cells (CRACC, SLAMF7) also belongs to the SLAM gene family and is expressed on cytotoxic T-cells, activated B cells, and mature dendritic cells [40]. Engagement of SLAMF7 activates NK cell-mediated cytotoxicity [40]. The mRNA level of SLAMF7 was highly expressed in ex vivo $CD28^-$CD8 memory T-cells and was stable after IL-15 treatment. Although the mRNA level of SLAMF7 was lower in ex vivo $CD28^+$CD8 memory T-cells, the level of SLAMF7 was similar between $CD28^-$ and $CD28^+$CD8 memory T-cells after IL-15 treatment.

Alteration of co-stimulatory receptors expression in $CD28^-$CD8 memory T-cells appears to be complex. While loss of expression of some receptors (CD28 and CD154) was apparent, elevated expression of other co-stimulatory receptors (4-1BB and SLAMF7) may provide a compensatory measure for the co-stimulatory function. The questions are: How are these different co-stimulatory receptors regulated in CD8 memory T-cells, particularly in $CD28^-$CD8 memory T-cells? Can an elevated expression of 4-1BB and SLAMF7 compensate the loss of CD28 and CD154? Further studies are required to address these issues and to better understand the activation associated defects of $CD28^-$CD8 memory T-cells and the mechanisms of the age-associated decline of immune function.

3.2.2 Expression of NK Cell Receptors in $CD28^-$CD8 T-cells

The NK cell receptors are initially identified on the surface of NK cells and NK T-cells. Based on their structures, NK cell receptors can be divided into (1) the Immunoglobulin-

like NK cell receptors including natural cytotoxicity receptors (NCR), killer immunoglobulin-like receptor (KIR), and CD244, and (2) the C-type lectin-like NK cell receptors including the killer cell lectin-like receptor (KLR) [41]. According to their functions, NK cell receptors can be divided into inhibitory and stimulatory receptors. While engagement of the inhibitory receptors prevents NK cells and CD8 T-cells from killing target cells, interaction of stimulatory receptors results in the trigging of NK cell or CD8 T-cell-mediated cytotoxicity. Expression of NK cell receptors on CD28⁻ CD8 T-cells have been reported [42]. Here we discuss the expression of seven NK cell receptors: KIR2DL2, KIR3DL2, NCR1, CD244, KLRD1, KLRF1, and KLRG1 during CD8 T-cell differentiation identified from microarray analysis.

KIR2DL2, KIR3DL2, NCR1, and CD244 belong to the Ig-like NK cell receptor family. KIR2DL2 (NKAT6) and KIR3DL2 (NKAT4) bind to the polymorphic MHC class I molecules and inhibits lymphocyte cytotoxicity. In contrast, NCR1 and CD244 are stimulatory NK receptors that activate NK-mediated cytotoxicity. The engagement of CD244 (2B4) with its ligand (CD48) or with an anti-CD244 antibody results in enhanced production of interferon gamma (IFN-γ) and cytotoxicity in CD8 T-cells and NK cells [43, 44]. Despite their opposite function, the expression patterns of these four NK cell receptors are similar: all of them are expressed in naïve cells, down-regulated in CD28⁺ memory T-cells, increased in CD28⁻ memory T-cells, and elevated in effector memory cells. After IL-15 treatment, all of them are down-regulated in CD28⁻CD8 memory T-cells [45]. As NCR1 is considered to be exclusively expressed in NK cells, the role of its elevated expression in effector memory T-cells remains to be determined. It has been shown that CD244 level is elevated in CMV-specific effector CD8 T-cells while absent in naïve CD8 T-cells [44]. This enhanced expression of CD244 in effector memory cells agrees with the elevated effector function of the CMV specific CD8 T-cells.

KLRD1, KLRF1, and KLRG1 belong to the C-type lectin-like NK cell receptor family. KLRD1 (CD94) forms heterodimers with KLRC3 (NKG2E) or other members. The KLRD1/KLRK1 (CD94/NKG2D) heterodimer is expressed primarily in NK cells and CD8 T-cells. KLRF1 (NKp80) is expressed in all NK cells and CD56⁺ T-cells and cross-linking of KLRF1 results in induction of cytolytic activity. KLRG1 is a newly identified member of the KLR family and is expressed in NK cells and a subset of T-cells. Although all of them are highly expressed in CD28⁻CD8 T-cells, the patterns of their expression differ. A down-regulation of expression from naïve to memory (CD28⁺) is observed for KLRD1 and KLRF1 but not for KLRG1. The identification of elevated expression of different NK cell receptors in CD28⁻CD8 memory T-cells suggests that NK cell receptors may play roles in CD28⁻CD8 T-cell function. The physiological significance of acquiring these NK cell receptors in CD28⁻CD8 T-cells is not clear and will require further study.

3.2.3 Expression of Cytolytic Molecules in CD28⁻CD8 T-cells

The function of cytotoxic CD8 T-cells is inducing rapid apoptosis of intracellular pathogen-infected or transformed cells. This cellular killing is mediated by two

distinct pathways: the granule exocytosis pathway that releases perforin and granzymes from the granule cores and the Fas ligand (FasL)/Fas pathway [46]. The granule exocytosis pathway consists of secretory granules that contain perforin and granzymes. Perforin is expressed only in cytotoxic T-cells and form a pore structure on the targeT-cell membrane to facilitate the entry of granzymes [47]. Granzymes are proteinases that consist of five members in humans: A, B, H, K and M. Each member of granzymes has a different substrate specificity [46]. Granzyme A (GZMA) and granzyme B (GZMB) are expressed in CD8 CTL, γδ T-cells, and NK cells [48, 49], granzyme H (GZMH) and granzyme K (GZMK) appear to be expressed mainly in CD8 CTL [50, 51], and granzyme M (GZMM) is expressed mainly in NK cells [52].

Perforin is detected in freshly isolated CD8 memory T-cells and is up-regulated after in vitro stimulation by TCR crosslinking or by treatment with IL15 [53]. The levels of perforin mRNA was higher in $CD28^-$CD8 memory T-cells than their $CD28^+$ counterparts [13, 45]. After culture with IL-15, there is no obvious increase of perforin mRNA levels in $CD28^-$CD8 memory T-cells but significantly increased perforin in $CD28^+$CD8 memory T-cells. The increase of proforin mRNA in IL-15 treated $CD28^+$CD8 memory T-cells is compatible to the level of freshly isolated $CD28^-$CD8 memory T-cells.

GZMB and GZMH share a high degree of similarity in amino acid sequence [50]. However, the expression patterns of GZMB and GZMH are quite different. The GZMB level is low in freshly isolated T-cells, but increases after activation [54]. The GZMH level is low in both freshly isolated and activated T-cells [55]. In $CD28^-$ CD8 memory T-cells, both GZMB and GZMH are highly expressed, resembling a mixed feature of activated T-cells and NK cells. GZMA and GZMK are functionally overlapping as up-regulation of GZMK has been found in GZMA deficient mice [51]. The levels of GZMA mRNA are similar between $CD28^-$ and $CD28^+$ CD8 memory T-cells, but a low level of GZMK is found in $CD28^-$CD8 memory T-cells compared to their $CD28^+$ counterparts. The level of GZMM expression was similar between $CD28^-$ and $CD28^+$CD8 memory T-cells. Following culture with IL-15, the expression of both GZMA and GZMB were increased in both CD8 memory T-cell subsets. Although activation by antigen do not significantly increase the expression of GZMH [55], IL-15 treatment induces up-regulation of GZMH in $CD28^+$CD8 memory T-cells. But $CD28^-$CD8 memory T-cells still have higher levels of both GZMB and GZMH than $CD28^+$CD8 memory T-cells. This indicates that $CD28^-$ CD8 memory T-cells possessed more cytolytic granule enzymes than $CD28^+$ counterparts before and after IL-15 culture, providing a molecular basis for high levels of cytotoxicity of $CD28^-$CD8 memory T-cells.

The Fas ligand (FasL)/Fas pathway provides another means of T-cell cytotoxicity, which applies not only to regular target cells such as intracellular pathogen infected and transformed cells but also to immune cells as a negative feedback regulation to the generation and expansion of CD4 and CD8 T-cells [46, 56]. It has been reported that FasL can block expression of CD28 at the transcriptional level in Jurkat cells [57], suggesting the role of FasL/Fas in the age-related decline of CD28 expression. The level of FasL mRNA appears higher in $CD28^-$CD8 memory T-cells than in

$CD28^+$ counterparts, and IL-15 treatment did not increase FasL expression in $CD28^-$ CD8 memory T-cells but increased in $CD28^+$CD8 memory T-cells. Together, the elevated expression of key molecules in both the granule exocytosis pathway and the FasL/Fas pathway indicates an enhancement of cytolytic capability in $CD28^-$ CD8 memory T-cells.

3.2.4 Expression of Cytokines, Chemokines and Their Receptors in $CD28^-$CD8 T-cells

Cytokines and chemokines are secreted proteins and play essential roles in many aspects of immune functions. In lymphocytes, cytokines or chemokines can promote their survival or death through strict regulation of their expression and their receptor expression during lymphocyte development and differentiation. Activation of lymphocytes induces production of a variety of cytokines and chemokines in turn these cytokines and chemocykes influence the effectiveness or determine the consequence of an immune response. Therefore, alteration of expression of cytokines and chemokines and their receptors could lead to mild or even severe defects of immune function [58].

Changes in cytokine and chemokine production in $CD28^-$CD8 T-cells after in vitro stimulation have been previously reported [10, 12, 59]. In freshly isolated $CD28^-$CD8 memory T-cells, the mRNA levels of interleukin 12A (IL12A), interleukin 13 (IL13), chemokine (C-C motif) ligand 4 (CCL4, MIP1-β), chemokine (C-X3-C motif) receptor 1 (CX3CR1, CCRL1), and chemokine-like receptor 1 (CMKLR1) are more highly expressed than do $CD28^+$CD8 memory T-cells. In contrast, the mRNA levels of interleukin 3 (IL3), interleukin 23A (IL23A), interleukin 7 receptor (IL7R), and interleukin 12 receptor β2 (IL12RB2) were more highly expressed in $CD28^+$CD8 memory T-cells compared with the $CD28^-$ cells.

IL-12 and IL-23 are cytokines that are composed of two subunits, one common subunit (IL12B, p40), and one unique subunit IL12A (p35) and IL23A (p19) for IL12 and IL23, respectively [60, 61]. Functionally, IL-12 induces the production of IFN-γ in NK and T-cells, facilitates Th1 differentiation, and serves as a bridge between non-specific innate resistance and antigen specific adaptive immunity [60]. In contrast, IL-23 participates in the proliferative signal in memory T-cells [60, 61]. At the mRNA level, IL12A is higher in $CD28^-$CD8 memory T-cells ex vivo but down-regulated after IL-15 treatment. In contrast, IL23A is highly expressed in $CD28^+$CD8 memory T-cells ex vivo, but was not changed after IL-15 treatment. The increased level of IL12 in $CD28^-$CD8 memory T-cells could contribute to the cytotoxicity of these cells while decreased levels of IL23A may affect the proliferative response of $CD28^-$CD8 memory T-cells.

IL-13 is produced primarily by Th2 cells and NK cells and promotes survival, differentiation, and proliferation of hematopoietic progenitor cells [62]. It also exerts immunoregulatory functions including anti-inflammatory effects, Th2 cell development, and B cell proliferation and IgE production [63]. By regulating cell-mediated immunity, IL-13 modulates resistance to several intracellular organisms [63]. IL13 mRNA level was higher in $CD28^-$ than in $CD28^+$CD8 memory T-cells

ex vivo. After IL-15 culture, IL13 mRNA level does not increase in CD28$^-$CD8 memory T-cells but increases in CD28$^+$CD8 memory T-cells. The precise role of IL-13 in CD28$^-$CD8 memory T-cells requires further study.

IL-7 is an essential cytokine during T-cell development and also plays a key role in homeostasis of memory CD8 T-cells [64]. IL-7 receptor is a dimmer that consists of IL-7 unique α receptor (IL7R) and the common γ chain. The function of IL7 depends on the expression of IL7R, which appears to be regulated in T-cells by the availability of IL-7 [65]. The mRNA level of IL7R is lower in CD28$^-$CD8 memory T-cells than in the CD28$^+$CD8 memory T-cells and is further down-regulated in both subsets after IL-15 treatment. These findings suggest that IL-7 may not be a key survival cytokine for CD28$^-$CD8 memory T-cells.

The primary function of chemokines is regulating lymphocyte migration but they are also involved in lymphocyte development, differentiation, and effector function. Like cytokines, different expression patterns of chemokines and their receptors were also observed between CD28$^-$ and CD28$^+$CD8 memory T-cells. CD28$^-$CD8 memory T-cells express higher levels of CCL4 (MIP-1β) and CX3CR1 compared to CD28$^+$ counterparts. Both are involved in the regulation of adhesion and migration of T-cells and NK cells [66, 67]. In addition, the expression of CX3CR1 is found in CTL and NK cells [68, 69]. Interaction of CX3CR1 with its ligand, CX3CL1 (Fractalkine), induces the adhesion function as well as promotes subsequent migration to the secondary chemokines such as CCL4 or IL-8/CXCL8 [69]. After IL-15 treatment, the mRNA levels of CCL4 and CX3CR1 are increased in both subsets of memory cells. In addition, the mRNA levels of chemokines XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β) are induced to significantly higher levels after IL15 treatment in CD28$^-$CD8 memory T-cells compared to their CD28$^+$ counterparts. Since they induce both T-cell and NK cell migration [70], elevated expression of XCL1 and XCL2 may facilitate migration of CD28$^-$CD8 memory T-cells. Three chemokine receptors, CCR2, CCR6, and CCR7, express more highly expressed in CD28$^+$CD8 memory T-cells than in CD28$^-$CD8 memory T-cells. After IL-15 culture, the mRNA levels of CCR2 and CCR6 are increased while the level of CCR7 was decreased in both subsets of memory cells.

3.2.5 Differentially Expressed Transcription Factors in CD28$^-$CD8 T-cells

The interaction between T-cells and other cells at various lymphoid compartments mediated by different ligands/receptors on the cell surface is an ongoing process throughout the life of T-cells. The consequence of these interactions depends on the specific interaction, the strength of the interaction, and the states of interacting cells, which are essential for the development, differentiation, and function of T-cells. One of the consequences of the surface ligand/receptor interaction is activation of transcription factors. Here, we will discuss four transcription factors that are differentially highly expressed either in CD28$^-$ (TBX21, EOMES, and MYC) or in CD28$^+$ (CEBPD) CD8 memory T-cells.

T-box 21 (TBX21, T-bet) is a member of T-box containing gene family and is involved in initiating Th1 lineage development from naive precursor cells and

regulation of Ig class switching in effector cells [71, 72]. In addition, it is also involved in regulation of the effector function by promoting IFN-γ production and cytotoxicity in CD8 T-cells [73, 74]. The level of TBX21 mRNA is higher in CD28⁻CD8 memory T-cells than in the CD28⁺ counterparts but there is no significant difference in the levels of IFN-γ and other Th1 cytokine genes between CD28⁻ and CD28⁺CD8 memory T-cells. Thus, it is plausible that elevated expression of TBX21 may serve as a regulator behind the elevated cytotoxicity in CD28⁻CD8 memory T-cells.

Eomesodermin (EOMES) is also a member of the T-box containing gene family within the same subfamily of TBX21. EOMES has been shown to induce IFNγ, perforin, and GZMB in CD8 T-cells [75]. The level of EOMES is higher in CD28⁻CD8 memory T-cells than in their CD28⁺ counterparts. After IL-15 treatment, the levels of EOMES are decreased only in CD28⁻CD8 memory T-cells but did in CD28⁺CD8 memory T-cells. As EOMES shares similar function with TBX21 in regulation of effector functions of CD8 T-cells, their elevated expression in CD28⁻CD8 memory T-cells provides a transcriptional basis of enhanced cytotoxicity in CD28⁻CD8 memory T-cells.

MYC and its family transcription factors are key regulators of cell growth and proliferation as well as inhibition of terminal differentiation and induction of apoptosis [76]. Dysregulation of MYC expression leads to unlimited cell growth and ultimately development of tumors [77]. MYC is up-regulated after T-cell activation and is also involved in the induction of apoptosis [78]. The level of MYC mRNA is higher in freshly isolated CD28⁻CD8 memory T-cells than their CD28⁺ counterparts. After IL-15 culture, MYC mRNA levels are highly increased in both CD28⁻ and CD28⁺CD8 memory T-cells. However, the difference of MYC mRNA level remained significantly higher in CD28⁻CD8 memory T-cells compared to their CD28⁺ counterparts. As previously studies showed CD28⁻CD8 memory T-cells are resistant to apoptosis, MYC in CD28⁻CD8 memory T-cells may facilitate cell division and resistant to apoptosis.

CEBPD is a member of the CCAAT/enhancer-binding protein (C/EBP) family that contains a highly conserved of leucine zipper DNA binding motif [79]. Members of the C/EBP family have been shown to regulate the differentiation of myelomonocytic marrow cells [80]. CEBPD is also involved in regulating the expression of IL6 that plays an important role in regulating immune and inflammatory response [81].The level of CEBPD mRNA is higher in CD28⁺CD8 memory T-cells than in CD28⁻CD8 memory T-cells. Although CEBPD expression is tightly regulated in G(0) growth-arrested mouse mammary epithelial cells (MEC) [82], IL-15 induced proliferation does not appear to affect the levels of CEBPD mRNA in either CD28⁻ or CD28⁺CD8 memory T-cells. It remains to be determined the significance of down-regulated expression of CEBPD in CD28⁻CD8 memory T-cells.

4 Conclusion

Studies of the generation of CD28⁻CD8 T-cells indicate that both antigenic stimulation and homeostatic proliferation are causes for loss of CD28 expression in CD28⁺ CD8 T-cells. Thus, it is likely that age-associated accumulation of CD28⁻CD8

T-cells is the combinational consequence of antigenic stimulation and homeostatic expansion of memory T-cells. Genome-wide analysis of gene expression profiles between $CD28^-$ and $CD28^+CD8$ T-cells reveals the molecular changes in $CD28^-$ CD8 memory T-cells. The gain and loss of these specific gene expressions in $CD28^-$ CD8 T-cells may reflect an adaptive process of the immune system in which an induced cytotoxicity is replaced by a constant cytotoxicity in CD8 memory T-cells in compensation of the inability of robust proliferation. Further characterization of the regulation and function of those differentially expressed genes in $CD28^-$ CD8 T-cells will help us to better understand this age-associated change in T-cell function and may open new avenues of clinical intervention to slow or reverse this aging-associated process.

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Role of Regulatory Subsets During Aging

Piotr Trzonkowski

Contents

Abstract: Efficient immune response requires both vigorous effector responses and regulation via regulatory subsets. Any disturbances in the balance between these two opposite activities of immune system result in either autoimmunity or excessive immunosuppression. Undoubtedly immunosenescence contributes to this balance as it affects the majority of populations taking part in immune response. This chapter describes activities of regulatory subsets, alterations associated with their ageing and clinical consequences of these changes.

Keywords: CD25+CD4+ Treg cells • Tr1 cells • Th3 cells • Interleukin 10 (IL10) • Transforming growth factor β (TGFβ) • Tolerogenic dendritic cells • CD28-CD8+ T suppressor cells • NKT cells

1 Introduction

Aggressive action of immune system against alien agents has to be strictly controlled in order to prevent destruction of self tissues. There are several subsets within lymphoid system which are responsible for the control of selective targeting of alloanti-

P. Trzonkowski (✉)
Laboratory of Experimental Transplantology
Department of Histology and Immunology
Medical University of Gdańsk
Ul. Dębinki 1, 80-211 Gdańsk, Poland
Tel.: 0048 58 3491430; Fax: 0048 58 3491436
E-mail: ptrzon@amg.gda.pl

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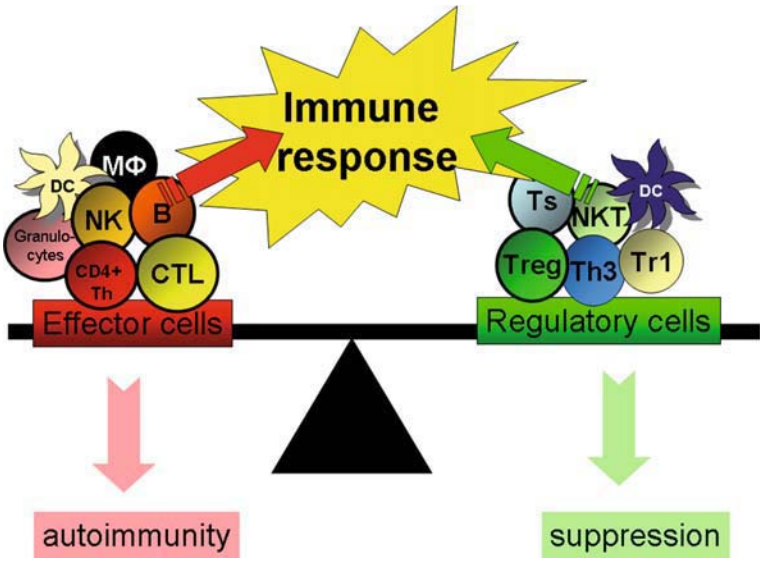

Fig. 1 The balance is required for efficient immune response. Coordinated function of effector and regulatory subsets guarantees efficient immune response. Imbalance in this regulation results is either autoimmune phenomena or exaggerated immunosuppression

gens and, at the same time, keeping the immune system neutral to autoantigens. The awareness of such immunosuppressive subsets started with Owen's notion, who found that the intraplacental transfusion of blood in cattle caused that each dizygote twin tolerated skin transplants from the other (Owen RD 1945). Soon after, Medawar performed a series of excellent Nobel Prize awarded experiments with infusion of alloantigens to newborn mice inducing selective alloantigen tolerance during their adulthood (Billingham RE 1953). The leading role of T-cells in tolerance induction was proved in 70s and 80s (Gershon RK 1970; Fujimoto S 1975; North RJ 1984); however, a detailed phenotype of those cells has been discovered only recently. In 1995, Sakaguchi reported that deficiency of CD4+ T-cells with expression of IL2Rα (CD4+CD25+), so-called T regulatory cells (Treg), in mice was associated with multiple autoimmune diseases (Sakaguchi S 1995). Without any doubt, different subsets of Treg cells have taken a central stage in immunology since that time. Nevertheless, induction of tolerance is much wider than Treg cells as some other subsets, such as CD28-CD8+ T suppressor cells (Ts), NKT cells, and some dendritic cells, were also found to confer it in immune system. Making the story even more complex, recent reports have suggested that efficient regulation of immune response is not limited to immunosuppressive cells, but it is more a balance between suppressive and proinflammatory effector cells. Interestingly, having opposite activities, at least some of those cells have common origin. Thus, keeping adequate proportions between aggressive effector phase of immune response and self-limitation of immune response is probably the best definition of the function of all the above mentioned regulatory subsets.

2 $CD25^{high}CD4^{+}$ T Regulatory Cells (Treg)

2.1 Biology

CD4+ T-cells play the central role in T-cell mediated regulation. There are two main subsets of CD4+ Treg cells in the body: naturally occurring and adaptive ones. As the discovery of Treg cells is relatively fresh, it is very often difficult to distinguish between these lineages due to their common features. Naturally occurring or intrinsic Treg cells (nTreg) in humans can be defined as $CD25^{high}$FoxP3+CD4+ T-cells. The expression of CD25 receptor is related to their high dependency on IL2, and FoxP3 is a transcription factor that drives intracellular signals which results in suppressive abilities of nTreg cells. Of note, FoxP3 is currently considered as the most characteristic intracellular marker of nTreg cells (Baecher-Allan C 2001; Hori S 2003). nTreg cells originate from the thymus. Maturing nTreg cells are self-reactive with intermediate to low affinity to autoantigens and yet they escape from central deletion. It is distinctive feature of their development in the thymus. Probably, they do not only arise as a result of the presentation of self antigens through TCR-dependent process but also by means of some other not well understood mechanisms. It has been only recently discovered that their intrathymic lineage commitment is maintained by interactions with medullary thymic epithelial cells expressing the autoimmune regulator AIRE (Aschenbrenner 2007). Intracellular mechanisms related to the maturation of Treg lineage are still far from final conclusions. The studies with *Scurfy* mice, that is, mice without active Treg cells due to the knockout of FoxP3 gene, revealed that these animals suffer from severe lymphoproliferative autoimmune disease (Fontenot JD 2003). Similar defect, so-called IPEX (IPEX–Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) was also found to be caused by mutations in FoxP3 gene in men (Bennett CL 2001). Genome-wide profiling revealed that transcription factor FoxP3 can bind to around 700 genes imposing phenotypic features of nTreg cells (Zheng Y 2007). Although FoxP3 takes a part in both differentiation and functioning of these cells, it appears that its action solidifies only pre-established features acquired by developing nTreg cells in the thymus as it has been shown that inactive nTreg may develop in the thymus even in the absence of FoxP3 (Gavin MA 2007; Wan YY 2007). Moreover, early stage of activation may be associated with transient expression of FoxP3 in T effector cells (Kretschmer K 2005). Despite self-specificity, nTreg do not damage own tissues at the periphery as they are highly anergic. The anergy might by explained by high differentiation of nTreg cells. It has been revealed that the expression of many surface markers locate them within memory phenotype, their proliferation is very much limited, they are characterised by short telomeres and easily undergo apoptosis (Taams LS 2001, 2002). The most important function of nTreg cells is the suppression of other immune cells. Autoreactive cells are not the only targets for nTreg cells. They are also highly efficient suppressors of alloresponses. Broad range of responses inhibited by nTreg cells caused that they were initially thought to be non-specific. However, more recent data, mainly from in vitro nTreg expansion experi-

ments, proved that their suppressive effect can be directed against responses driven by specific antigens (Masteller EL 2006). This inconsistency might have come from low affinity of their receptors and the phenomenon of bystander regulation exerted by nTreg cells, *i.e.* nTreg cells specific to particular antigen may impose tolerance to other antigens when activated (Waldmann H 2006). At first, only CD4+ T effector cells were described to be inhibited by nTreg cells (Suri-Payer E 1998). Twelve years after the discovery Treg cells were found to interfere with CD8+ T-cells, NK cells, NKT cells, monocytes, dendritic cells, and granulocytes (Piccirillo CA 2001; Trzonkowski P 2004; Taams LS 2005; Lewkowicz P 2006). Upon stimulation, which usually occurs at the site of inflammation as well as in the local lymphoid tissue, nTreg interact with effectors in a direct cell-to-cell manner suppressing their proliferation and effector activities (Taams LS 2001; Trzonkowski P 2004). Although nTreg cells were found to produce suppressive cytokines, such as IL10 or TGFβ, direct contact with other cells is regarded as the most important way of their action. The most important receptor of Treg cells cooperating in the immune synapse with both CD4+ and CD8+ T effector cells is CTLA-4 molecule (Cytotoxic T lymphocyte antigen 4, CD152). Engagement of this receptor in the presence of TCR ligation triggers suppressive activity of nTreg cells (Takahashi T 2000; Sansom DM 2006). There are several mechanisms of this suppression. Initially, it was postulated that nTreg cells may physically interfere with the interaction of effector T-cells with APCs by competing for the costimulatory molecules on APCs (Takahashi T 2000). It might be possible as, in comparison to the ligands expressed on T effectors, CTLA-4 has higher affinity for B7 family receptors on APC (Linsley PS 1992). In addition, nTreg cells express variety of adhesive molecules, such as ICAM-1 (CD54) and integrins LFA-1 (CD11a/CD18), α4β7 (LPAM-1), αEβ7 (CD103) and α4β1 (CD49d/CD49), that may additionally give Treg cells the advantage of cell-to-cell interaction with APC that is stronger than the interaction of APC with effectors (Takahashi T 2000; Stassen M 2004; Marski M 2005). Another explanation is that the interaction between CTLA-4 on nTreg cells and B7 family receptors on APC, notably in dendritic cells (DC), induces the expression of enzyme indolamine 2,3-dioxygenase (IDO) in the latter (Grohmann U 2003). IDO changes metabolic pathway of tryptophan to kynurenines, which suppresses T-cell responses (Mellor AL 2002). It has been also described that the engagement of CTLA-4 on nTreg induces secretion of TGFβ that subsequently strongly suppresses T effector cells (Chen W 1998). Apart from T-cells, cell-to-cell interactions were also described in the regulation of other immune cells by nTreg cells. Membrane-anchored TGFβ on nTreg cells is crucial for the inhibition of NK cells and TLR receptors expressed on nTreg cells are prerequisite for the regulation of the activity and survival of granulocytes (Ghiringhelli F 2005; Lewkowicz P 2006). The competition between nTreg cells and T effectors is not limited to the binding of surface receptors of APC. nTreg cells are highly dependent on IL2 but devoid of capabilities of its production. Thus, they compete with effector cells for IL2 which decreases the amount of IL2 available at the site of immune reaction and therefore tempers activities of T effectors (Thornton AM 1998). Moreover, despite dependency on IL2, Treg cells suppress production of IL2 by CD4+ T effectors which additionally decreases availability of

the cytokine during immune response. The most extreme pathway of the regulation revealed during research upon nTreg cells is their cytotoxicity. Namely, it has been found that these cells upon stimulation kill autologous cells by means of secreted cytotoxic perforin and granzymes (Grossman WJ 2004; Gondek DC 2005).

Peripherally induced or adaptive Treg cells constitute another subset of CD4+ Treg cells. It is a small number of cells generated during each and every immune response from activated naïve CD4+ T-cells at the periphery (Karim M 2004). These cells are antigen-specific as they arise in response to specific antigens and their activation is dependent on expressed TCR receptors. Importantly, it is a source of Treg cells independent of the thymus. Apart from that, basic characteristics of these so-called adaptive Treg cells, including the most important marker FoxP3, are similar to thymic-derived nTreg cells (O'Neill 2004).

There are also some other subsets of adaptive CD4+ Treg cells induced at the periphery. In contrast to nTreg cells, these cells do not suppress in cell-to-cell mode and their action is dependent mainly on secreted suppressive cytokines. Based on secreted cytokines at least two different groups may be distinguished—Tr1 cells, which function relies on secreted IL10 (Groux H 1997), and Th3 cells, which produce mainly TGFβ (Fukaura H 1996). Apart from cytokine-dependent mode of suppression, their basic characteristics are also different from nTreg cells. Tr1 cells are anergic mainly due to autocrine action of IL10. These cells seem to be dependent on IL2 family of cytokines as they constitutively express high levels of IL2 family receptors IL2Rβ (CD122) and IL2Rγ (CD132) and can be expanded in the presence of IL2 and IL15. On the other hand, normal level of IL2Rα (CD25) on Tr1 cells can be achieved only upon TCR-mediated stimulation (Battaglia M 2006). Also the expression of FoxP3 in Tr1 is not constitutive but can be upregulated upon activation (Vieira PL 2004). The most consistent intracellular protein postulated as a marker of Tr1 cells is the repressor of GATA-3 (ROG); however, its expression was also noted in T effectors (Cobbold SP 2003). The most important inducer of IL10-producing Tr1 are immature DC (Levings MK 2005). IL10 secreted during interaction of Tr1 with DC limits the production of IL12 and TNFα by DC and macrophages which subsequently quenches induction of Th1 and Th2 responses (Moore KW 2001). Tr1 cells were found to promote tolerance to both auto- and alloantigens. Their suppressive role was described in allotransplantations of bone marrow and solid organs, down-regulation of immune responses in rheumatoid diseases and other autoimmune pathologies, allergies and, inflammatory bowel diseases. On the other hand, their deficit was found to facilitate chronic course of some infections (Battaglia M 2006). It might be important in ageing that IL10 is secreted not only by Tr1 cells but also by other T-cells, monocytes, macrophages and nonlymphoid cells. IL10 from all those sources is often treated as a counterbalance to proinflammatory cytokines, notably to IL6 (Saurwein-Teissl M 2000; Ye SM 2001; Hacham M 2004).

Like Tr1 cells, TGFβ-producing Th3 cells are distinctive in several aspects. First of all, their function is linked mainly to oral tolerance. This aspect is of great importance as Th3 cells generated with orally administered antigens might exert bystander regulation, which has implications in pathology as well as in potential

therapeutic strategies (Ochi H 2006). Unlike other Treg cells, the generation of Th3 cells is dependent on IL4 (Fukaura H 1996; Hafler DA 1997). Also TGFβ on its own, or augmented by IL10, may generate Th3 cells from naïve T-cells (Chen W 2003; Kitani A 2003). Immunosuppressive action of TGFβ secreted by Th3 cells is directed against Th1 responses as it downregulates expression of IL12R and transcription factor T-bet in Th1 cells (Kitani A 2000; Gorelik L 2002). TGFβ may work as soluble cytokine but also as membrane-anchored receptor. The action of the latter form, in relation to TGFβ type 1 on Treg cells, was initially described as an important tool of cell-to-cell regulation of T effectors, B cells (Nakamura K 2001) as well as NK cells (Ghiringhelli F 2005). Membrane-anchored TGFβ seems to work as an executor of several pathways of regulation as its function can be activated by several factors, for example, latency-associated protein (LAP) or thrombospondin (Faria AM 2005). Like the system associated with IL10, TGFβ-dependent regulation appears to be much wider than Th3-mediated effects. The cytokine is fully capable of suppression of T-cells responses when produced by nonlymphoid lineages, such as macrophages or enterocytes (Barnard JA 1993; Galliaerde V 1995).

2.2 Ageing

The regulation of immune responses through Treg cells in ageing appears to have some distinctive features. When compared to younger subjects, the elderly are characterised by higher number of Treg cells but per-cell activity of those cells seems to be altered.

Several laboratories reported increased frequency of Treg cells in aged individuals (Trzonkowski P 2003; Gregg R 2005; Gottenberg JE 2005). The percentage of $CD25^{high}CD4+$ T-cells in the peripheral blood is surprisingly high at birth reaching in some cases even 9.5% of total CD4+ T-cells in cord blood (Godfrey WR 2005), but then decreases during childhood and remains on a stable level not exceeding 5% of total CD4+ T-cells in young and middle aged subjects (Cao D 2004; Beyer M 2005; Gottenberg JE 2005). In more advanced age the number of Treg cells gradually increases and, in the extreme, it may be even fivefold higher than that noted in earlier phases of ontogeny (Trzonkowski P 2006). There might be several sources which give rise to the increased number of Treg cells with ageing. First of all, longer life means longer time when the cells can be generated. Although thymic involution causes reduced output of naturally occurring Treg cells with age, adaptive Treg cells may be generated continuously at the periphery throughout entire lifespan. To a great extent, this idea was confirmed in animal model. Shimizu's group found that age-associated increase in FoxP3+ T-cells with regulatory properties was mainly attributed to CD25-CD4+ T-cells and not to the classical thymus-derived nTreg cells (Shimizu J 2003). In humans, FoxP3+ Treg cells were shown to arise from rapidly dividing, highly differentiated memory CD4+ T-cells (Vukmanovic-Stejic M 2006). Since both memory and regulatory subsets in particular subjects were revealed to share the same TCR repertoire, the authors of this report concluded that every chal-

lenge with specific antigen generates both memory and regulatory cells. This finding, consistent with other reports (Cobbold SP 2006), is of special importance in quantitative studies upon Treg cells in aging as it gives a link between the number of immune responses and potency of regulation in particular subject. It seems to be logic that aged individuals have had higher chance to be challenged with higher number of pathogens than the young, simply because they live longer. Thus, as it comes from Akbar's studies, the elderly are not only characterised by increased number of memory cells but also those regulatory (Vukmanovic-Stejic M 2006). Consistently with this view, when the number of Treg cells was compared between subjects of the same age, the higher number was found in those with the history of more frequent inflammatory events and exposures to higher number of antigenic challenges (Trzonkowski P 2003). Thus, it is not surprising that frail elderly with inflammatory burden (Pawelec G 2005) are characterized by higher number of Treg cells than their healthy counterparts (Trzonkowski P 2006). Preferential accumulation of Treg cells in unhealthy individuals may be recognized as a kind of "vicious circle," when Treg cells arising during particular responses make the patient more susceptible to subsequent infections and these infections induce more Treg cells. It may be especially detrimental in aged subjects as their "vicious circle" lasts for a long time and therefore the number of Treg cells is exceptionally high in frail elderly (Trzonkowski P 2006).

It has to be highlighted that the estimation from peripheral blood might not correlate with total number of Treg cells in the body as Treg cells are capable of efficient trafficking through lymphoid tissues where their level may be substantially higher than that measured in the peripheral blood. For example, Treg cells in mice were found to constitute 40% of CD4+ T-cells in the bone marrow or even more, in terms of absolute numbers, in the spleen (Hoffmann P 2002). Moreover, the trafficking through the tissues seems to be crucial for the function of Treg cells as the expression of receptors allowing them to enter lymphoid tissues, such as CD62L or CCR7, was associated with higher capabilities of immunosuppression (Fu S 2004; Taylor PA 2004; Ermann J 2005). Since the expression of these receptors declines with age on T-cells, it might be the reason that Treg cells in the elderly are not capable of extravasation and their level is increased in the peripheral blood but not in the tissues. These receptors are also markers of naïve cells which implies that Treg cells, like other T-cells, may be on different levels of their differentiation. Indeed, "naïve" CD45RA+FoxP3+CD4+ T-cells, which characteristics are close to other naïve T subsets, was described as a subset of Treg cells in humans. The percentage of these cells was shown to be substantially reduced with age (Valmori D 2005). Initially, no difference in ex vivo suppressive activity was found between CD45+ and CD45RA- Treg cells (Valmori D 2005; Seddiki N 2006). Nevertheless, it might have been dependent on incomplete phenotyping and assessment based on single sampling of the probands. More recent study, in which authors followed phenotype of Treg cells over time during ex vivo expansion, proved superiority of Treg cells derived from CD45RA+ precursors above those CD45RA- (Hoffmann P 2006).

Bearing in mind that the majority of Treg cells in the elderly are highly differentiated and their development, to some extent, is parallel to memory/effector cells, it

might be possible that their accumulation might be an attempt of the counteraction to the process of "shrinkage and filling up of the immunological space" hypothesized initially by Franceschi's group (Franceschi C 2003). The theory is based on the observation that lymphoid system in aged individuals is filled with expanded clones of anergic CD8+ T-cells that block proper immune responses to new challenges (Ku CC 1997). Homeostatic proliferation seems to be an important phenomenon responsible for the generation of CD8+ T clones during ageing (Ku CC 2000; Surh CD 2000; Goronzy JJ 2007). The process has not yet been fully understood but it is known that it is regulated by homeostatic cytokines IL15, IL7, CCL19, CCL21, and MHC-signaling which allow T-cells for rapid expansion in the absence of any external stimuli. Interestingly, recent reports have proved that Treg cells are important players in the limitation of homeostatic proliferation of nonregulatory T effector cells (Shen S 2005). Moreover, Treg cells do not undergo homeostatic expansion on their own (Liu W 2006; Seddiki N 2006). In the light of these facts, their accumulation with age might be surprising as the logic indicates that homeostatic proliferation should preferentially give rise to the increased number of CD8+ T clones and, at the same time, keep the number of Treg cells low. The explanation comes from the nature of resistance of Treg cells to the homeostatic mechanism. Namely, Treg cells are devoid of the expression of IL7R (CD127; Liu W 2006; Seddiki N 2006) and therefore, in contrast to other CD4+ T-cells, they do not require IL7 for survival. The level of this homeostatic cytokine declines with age together with the shrinkage of its main producer, the stroma of the thymus and other lymphoid organs (Fry TJ 2001; Aspinall R 2002). Thus, independence from IL7 may be the reason that CD4+ Treg cells, in contrast to non-regulatory CD4+ T-cells, accumulate with age. Making the image complete, CD8+ T clones are able to accumulate in a homeostatic manner even more vigorously than Treg cells because they are not very much dependent on IL7 and utilize IL15 instead (Chiu WK 2006). Importantly, IL2 is prerequisite for Treg cells to inhibit homeostatic proliferation of other T-cells (Murakami M 2002). In this regard, the role of Treg cells in the suppression of homeostatic proliferation might be somewhat ambiguous as, on the one hand, Treg cells require IL2 for the limitation of homeostatic proliferation of other cells but, on the other hand, they inhibit production of this cytokine by CD4+ T effectors (Piccirillo CA 2001). It might be possible that beyond some threshold the accumulation of Treg cells may be the reason of self-limitation of their activity due to deprivation of IL2. Reaching this point, Treg cells no longer prevent from homeostatic proliferation of T effectors. Indeed, frail elderly seem to "cross the threshold" as they are characterized by extremely high number of Treg cells concomitantly with deep deficiency of IL2 (Trzonkowski P 2006). It seems that the regulation of homeostatic proliferation by Treg cells was designed by the evolution for short-living individuals. Long-lasting or repetitive stimulation, such as continuous stimulation with pathogens like CMV described widely during ageing, might be capable of destabilization of this regulatory circuit which subsequently results in detrimental expansion of oligoclonal CD8+ T-cells found preferentially in frail elderly.

It is not a long time since the discovery of Tr1 and Th3 cells was made and therefore the data specifically on these two subsets in ageing are scarce yet. Up to date,

many questions related to these two suppressive subsets of CD4+ T-cells remain not addressed. In many cases, it is not possible to split up the function of different regulatory subsets of CD4+ T-cells in a given experimental model. Performed studies very often suggest overlapping between phenotype and function of different subsets. The main suppressive cytokines, TGFβ and IL10, are not only secreted by CD4+ T-cells but also by other lymphocytes and nonlymphoid cells and they do not only exert action on immune system but also on other tissues. IL10, in particular, is associated with regulation in ageing as it is often contrasted with proinflammatory activities of IL6 and other proinflammatory cytokines reported to be overexpressed in the elderly. Polymorphic variant -1082GG of IL10 gene, which is associated with high production of IL10 (Persico M 2006), was found to be preferentially spared in centenarian males (Lio D 2002). Interestingly, animal studies suggest surprisingly well preserved secretion of IL10 in old animals versus young ones in epithelial organs such as intestine and kidney (Hacham M 2004). This finding is consistent with previous reports that IL10, also secreted by Tr1 cells, modulate preferentially mucosal immune responses (Nakagome K 2005; Uhlig HH 2006). The cytokine secreted by Tr1 cells might be also involved in aging of cardiovascular system. High levels of IL10 were found in hearts from old mice during their healthy ageing (Hacham M 2004). Increased expression of IL10 in the wall of aorta after adoptive transfer of Treg cells was found to be an agent slowing down atherosclerosis in apolipoprotein E-knockout mice (Mor A 2007). Involvement of IL10 in the circulatory system was also described in humans where low levels of IL10 were associated with complicated recovery after coronary artery bypass grafting (Wei M 2003). Some other studies did not find age-related differences in the levels of IL10 but pointed at increased levels of soluble form of TGFβ in plasma of the elderly (Forsey RJ 2003). Increased secretion of TGFβ type 1 was revealed in response to elevated levels of IL6, being a counterbalance to proinflammatory activity of the latter (Villiger PM 1993). Both TGFβ type 1 and 2 were found to interfere with IL7 in thymopoiesis which might contribute to faster involution of the thymus (Chantry D 1989). At the periphery, TGFβ was described to suppress many different cells. It seems that apart from inhibitory action on T-cells (Letterio JJ 2000), TGFβ is involved in the inhibition of macrophages, which might be of great importance in the prevention of inflammageing (Erwig LP 1998). High levels of TGFβ in aged individuals appear to be consistent with increased expression of CTLA-4 on T-cells reported in this age group (Wakikawa A 1997; Leng Q 2002). Reciprocal interrelation between TGFβ and CTLA-4 may have some functional implications. Cross-linking of CTLA-4 results in the secretion of TGFβ by CD4+ T-cells (Chen W 1998). On the other hand, TGFβ accelerates the expression of CTLA-4 on T precursors which facilitates transformation of CD4+CTLA-4+ T precursors to adaptive Treg cells (Zheng SG 2006). As mentioned above, TGFβ secreted by regulatory cells must be separated from other sources as, for example, locally decreased production of this factor by fibroblasts was linked to impaired wound healing in the elderly (Kudravi SA 2000). Moreover, soluble form of the cytokine is significantly less functional than that membrane-bound and the level of the latter was found to drop down quite early in life in animal model (Gregg RK 2004). Nevertheless, we still

lack the knowledge about age-related differences in the expression of membrane-bound TGFβ in humans.

Despite many efforts, there is no certainty that the accumulation of Treg subsets with age results in oversuppression of the immune system in the elderly. For example, inflammageing, one of the most commonly accepted theories of ageing, contradicts exaggerated immunosuppression in the elderly. Indeed, some reports suggest that the activity of Treg cells declines with age (Tsaknaridis L 2003). Importantly, these studies compare suppressive ability of equal numbers of highly purified Treg cells sorted from either elderly or young subjects in various in vitro suppression assays. In fact, lower responsiveness of Treg cells from the elderly, as compared to the young, in these tests may simply illustrate an impairment of aged Treg cells on a per-cell basis. If the quality of single Treg cell taken from aged subject is not as good as that from the young, it is not surprising that the same number of Treg cells taken from the elderly and the young does not reveal similar suppressive abilities in the assay. The suggestion that per-cell Treg activity from older subject is lower than that from the young is of great importance in the light of reports that local rather than systemic level of Treg cells is associated with clinical outcomes (Liu W 2006). As such, small proportion Treg cells trafficking to the place of local inflammation, might be effective enough in the young but insufficient in the elderly. Bearing in mind this quality issue, the accumulation of Treg cells reported in the elderly may be not necessarily associated with high suppressive abilities but rather recognized as a compensation for their per-cell impaired functioning. Indeed, altered phenotype of Treg cells with age, manifested as low proportion of CD45RA+ naïve Treg cells, seems to prove this hypothesis. Another example of impaired "molecular hardware" of aged Treg cells is their inability to undergo apoptosis. It was described mainly in frail elderly and was recognized as a reason of Treg cell accumulation with age (Trzonkowski P 2006). Of note, although the level of Treg cells was revealed to be extremely high in frail elderly, they were unable of efficient action as those patients were the most affected by detrimental effects of inflammageing (Trzonkowski P 2003). It clearly indicates that aged Treg cells are somehow defective. It is possible that prolonged exposure to environmental factors throughout the lifespan might be responsible for defects of Treg cells. Such environmental influence in the elderly was already described as a cause of damage of naïve CD4+ T-cells (Haynes L 2002).

Although accumulation of Treg cells was not found to be faster in any gender, some specific physiological milestones of human life were linked with sudden increase or decrease in the suppressive activity of Treg cells, notably per-cell Treg cell activity. The best described effects are associated with pregnancy when Treg mediated suppression increases in order to tolerate foetal tissues (Aluvihare VR 2004; Somerset DA 2004). However, slightly increased suppressive activity can be also detected during each and every luteal phase of menstrual cycle being interpreted as an action facilitating implantation of the embryo (Mysliwska J 2000; Trzonkowski P 2001). It is very likely that protolerant action of Treg cells towards embryonic tissues is the most pronounced locally. For example, primary idiopathic infertility is associated with low density of Treg cells in endometrium (Jasper MJ

2006). Obviously, the activity of Treg cells in these phenomena is driven by sex hormones, notably oestradiol (Prieto GA 2006). No surprise, fading hormonal activity around menopause is associated with loss of per-cell Treg activity that tips the effector/suppressor balance in favor of the former (Rachon D 2002; Arruvito L 2007). For example, a peak of some Th1-dependent autoimmune diseases associated with menopause in women, such as rheumatoid arthritis, might be triggered by decreased per-cell activity of Treg cells (Ehrenstein MR 2004). Somehow similar effects, but less clear, were also described for androgens (Page ST 2006). Importantly, also other steroid hormones, both endogenous and administered as drugs, are known to keep proper physiological activity of Treg cells (Fattorossi A 2005).

In general, the discussion about suppression in immune system should take into account effector/suppressor balance rather than suppressors only. It might be of special interest in the elderly, where the activation of immune system is prolonged and elevated. Namely, it was found in animal model that Treg cells generated throughout life regulate weak to moderate immune responses mediated by T effector cells. On the other hand, strong stimulation of T effector cells could not be stopped by endogenous Treg cells and only adoptive transfer of relatively high number of Treg cells specific to the stimulus was able to limit the response (Billiard F 2006). Are these conditions adaptable in humans? Would it be possible that we are able to control our immune responses to some level and when the input of activatory signals is too high or too long, like in the case of inflammageing and CMV, endogenous Treg cells are no longer capable of control over T effectors? Some experimental data answers in the affirmative. Some factors associated with inflammation may turn the effector/suppressor balance in favor of exaggerated effector responses. Proinflammatory cytokines, such as TNFα and IL6 were described as strong inhibitors of Treg cell function (Valencia X 2006; Wan S 2007). Bearing in mind that inflammageing is associated with extremely high production of TNFα and IL6, it is not surprising that above some threshold Treg cells in the elderly are no longer able to counteract inflammation. Moreover, recently discovered strong proinflammatory subset of CD4+ T-cells, Th17 cells, were found to have common ties with Treg cells on a very early stage of development. Th17 cells exert their actions mainly through secreted members of IL17 family cytokines, which are very strong stimulators of inflammatory responses (Veldhoen M 2006). In the extreme, as it comes from animal models, they may be involved in the development of chronic inflammation and autoimmune diseases (Romagnani S 2006). Pathways leading to transition of precursor cells to either Treg cells or Th17 cells were proved to have some common features. Like in the case of adaptive Treg cells, the generation of Th17 cells requires TGFβ (Bettelli E 2006; Veldhoen M 2006). However, the transition to Th17 cells needs also the addition of IL6. The secretion of IL6 by DC stimulated with lipopolysaccharide, in the presence of TGFβ, was necessary to generate Th17 cells. Interestingly Treg cells could be a source of TGFβ in this process and secretion of TGFβ by Treg cells in the presence of IL6 results inevitably in the generation of Th17 cells and not Treg cells. Other inflammatory stimuli, TNFα and IL1, were not necessary but strongly enhanced this process (Veldhoen M 2006). Moreover, activated Treg cells could themselves differentiate into Th17 cells in the presence of IL6 (Xu L

2007). This mechanism of regulation is of special importance in the elderly, where inflammageing phenomena may easily add inflammatory stimuli to those secreted by Treg cells and generate Th17 cells and disturb effector/suppressor balance. Ironically, accumulation of Treg cells in frail elderly would not prevent from inflammageing but rather gave more signals necessary to generate Th17 cells.

2.3 Infectious Diseases

The accumulation of Treg cells with age is an attractive explanation of commonly known susceptibility to infections and high incidence of some tumours among the elderly. However, regulation mediated by suppressive mechanisms should not be always treated as a "pure evil." Effective regulation seems to be inevitable to focus immune response on the pathogen clearance and not on unnecessary exaggerated inflammation leading to destruction of self tissues. For example, lack of IL10-dependent regulatory circuit, as shown in IL10-deficient mice, was responsible for high sensitivity to lysteriosis (Deckert M 2001), gram-negative peritonitis (Sewnath ME 2001) or chronic active *Helicobacter pylori* gastritis (Chen W 2001). TLR4-defective mice were also found to be highly susceptible to infection with *Bordetella pertussis* due to low production of IL10 (Higgins SC 2003). In all those models immune response was fulminant and led to a damage of self tissues and not to elimination of invading pathogen.

Of note, TLR-dependent activity is the example of yet another way of regulation of antipathogen responses by Treg cells. It has been only recently found that Treg cells are able to directly sense pathogens through expressed TLR receptors (TLR4, TLR5, TLR8 in humans; Caramalho I 2003). Signals received by Treg cells through at least some of those receptors (TLR2 in mice and TLR8 in humans) were found to decrease suppressive abilities of those cells at the time of acute infection which allowed for effective immune response. However, the same signals promoted proliferation of Treg cells. Thus, it is hypothesized that TLR-dependent regulation makes the activity of Treg cells low during pathogen clearance and, at the same time, promotes the generation of expanded clones of Treg cells that attenuate potentially harmful responses of residual T effectors left when the infection is gone (Peng G 2005; Sutmuller RP 2006). The delay in TLR-stimulated activity of Treg cells, as compared to other subsets regulated by TLR stimulation, comes from the fact that Treg cells need much stronger signals (more microbial products) than other immune cells to be activated via TLR receptors (Raghavan S 2005). In the light of this mechanism, repetitive infections may explain accumulation of suppressive Treg cells with age. It might be relevant in the clinic as some authors tempt to speculate that the protection from atopic diseases in adults might be linked to increased frequency of regulatory cells generated during frequent infections with some pathogens during childhood (Braun-Fahrlander C 2002; Yazdanbakhsh M 2002). It is possible that it is yet another example of "short-sightedness of evolution," when the mechanism good for young individuals might be disastrous in the elderly. At some

point, accumulation of Treg cells may become detrimental as it leads to insufficient responses to new infectious challenges, which results in chronic nature of infections in the elderly or, in the worst case, can prove fatal.

As already mentioned, Treg cells are capable of inhibition of the variety of immune cells. The suppression of effector cells, when affects somehow deteriorated T-cells in the elderly, might be a reason of too weak effector responses. Looking into the spectrum of cells suppressed by Treg cells it is not surprising that Th1 responses are the most depressed. Treg cells suppress efficiently cells with abilities to exert cytotoxic effect, such as CTL, NK and macrophages. Thus, infections that are cleared by cellular type of immunity, where cytotoxic activity is crucial, are more difficult to control in patients with increased activity of Treg cells. It is very much important in the elderly. Treg cells, mainly nTreg cells, make infections chronic by decreasing cytotoxic abilities of effectors as revealed in persistent infections caused by *Hepatitis C virus, Herpes simplex virus* and CMV (Boettler T 2005; Vahlenkamp TW 2005). Involvement of those cells was also described in some other infections known to be associated with deteriorated immunity in ageing, such as candidiasis (Netea MG 2004), tuberculosis (Chen X 2007) or pneumocystis pneumonia (McKinley L 2006).

2.4 Tumors

Increased frequency of tumors is recognized as another consequence of increased frequency of Treg cells in the elderly (Sharma S 2006). The presence of Treg cells associated with tumors might be extremely dangerous in aged individuals as a high proportion of those cells recognize self-antigens. As such, these cells mediate tolerance also to aplastic self tissue of growing tumors, which additionally hampers immune response compromised already by ageing. Accumulation of Treg cells was revealed in a wide variety of tumors of different origin (Betts GJ 2006). Some of the studies managed even to correlate increasing level of Treg cells with disease progression and patient survival (Curiel TJ 2004; Wolf D 2005). Importantly, high number of the studies reported increased number of Treg cells in cancer, predominant type of tumors in the elderly (De Pinho RA 2000). The most convincing link between Treg cells and tumor immunity comes from the studies on NK cells. NK cells are one of the most important elements of immune surveillance against tumors. It is remarkable that aged individuals characterized by low activity of NK cells are at higher risk of cancer onset when compared to those with high NK activity (Imai K 2000). It has been described that Treg cells, mainly nTreg cells, are strong inhibitors of NK cells (Trzonkowski P 2004). Utilizing membrane-bound TGFβ, Treg cells inhibit cytotoxic activity of NK cells in the site of tumor as well as in local lymph nodes and peripheral blood. Importantly, the effect appeared to be quite universal as it was found against gastrointestinal stroma tumors (GIST), melanoma, different types of cancer and, leukaemia cells in both humans and animals (Wolf AM 2003; Ghiringhelli F 2005; Smyth MJ 2006). Apart from NK cells, also CD8+ T-cells were found to be directly inhibited by Treg cells within the tumor mass (Curiel TJ 2004).

It might be also possible that tumor environment on its own is able to induce regulatory cells from naïve precursors. For example, it is widely known that many tumors secrete TGFβ necessary to transform naïve T-cells to Treg cells. Consistent with this finding, tumor-infiltrating Treg cells are mainly Tr1 and Th3 cells which suppress effector cells via secreted cytokines (Chacrabarty NG 1999; Liyanage UK 2002). Moreover, some tumor cells and tumor-infiltrating inflammatory cells secrete chemokines, such as CCL22, attracting Treg cells to the site of tumor (Curiel TJ 2004).

Some anti-tumor drugs were confirmed to have an impact on Treg cells. Small dose of cyclophosphamide was confirmed to induce selective apoptosis of Treg cells in humans and animals (Ghiringhelli F 2004; Lutsiak ME 2005). Similar effect was also described after administration of another oncological drug, fludarabine (Beyer M 2005). There are also attempts of more specific immunotherapy targeting Treg cells. For example, depletion of those cells resulted in a better immune response to tumors or when performed prior to the administration of ant-itumor vaccines, enhanced effects of anti-tumor vaccines against cancer cells in animals (Dannull J 2005; Nair S 2007). It is of special importance in the elderly as the effectiveness of anti-tumor vaccines in preclinical models was revealed to be low at this age and there are suggestions that age-associated accumulation of adaptive Treg cells might have been responsible for this effect (Gravekamp C 2007).

2.5 Autoimmunity

The role of Treg cells in the elderly is intriguing when autoimmunity is taken into account. It is surprising that there is an accumulation of Treg cells and, at the same time, the incidence of autoimmune phenomena during ageing is higher than during earlier ontogeny (Stacy S 2002). Nevertheless, there are few characteristic features of autoimmunity in aged individuals, which may explain this apparent paradox. First important feature is the hormonal introduction to senescence. Menopause, and less evident andropause, are associated with a peak of incidence of some autoimmune diseases, mainly those Th1-dependent like rheumatoid arthritis or Hashimoto's thyroiditis. As already mentioned, lack of hormonal protection starting with menopause (and to a lesser extent with andropause) might be associated with a decrease in regulatory function of Treg cells which facilitates the onset of such disorders (Arruvito L 2007). Later phases of senescence are more associated with Th2-dependent autoimmunity. Shrunk naïve compartment and cytokine balance skewed towards Th2 cytokines makes B memory cells the leading cause of autoimmune phenomena in the elderly. Of note, the control provided by Treg cells over B cells, notably over B memory cells, is very much limited. It is rather indirect suppression as Treg cells regulate mainly CD4+ T helper cells cooperating with B cells (Guay HM 2007). Moreover, as B memory cells are less dependent on signals from CD4+ T helper cells than their naïve precursors, therefore B memory cells are the least affected by Treg cells. Hence, a wide variety of autoantibodies can be found

in the elderly (Stacy S 2002). Streaking feature of these autoantibodies is that the vast majority of them is not linked to any autoimmune disease and seem to be not interfering with the health status (Xavier RM 1995; Nilsson BO 2006). It might be possible that, like during earlier life, crucial immunodominant self antigens are still protected from autoaggression in the elderly. Obviously, accumulation of Treg cells is relevant for this regulation but it is not the only event contributing to this phenomenon (Specht C 2003). Increased proportion of CD5+ B1 cells and elevated level of antiidiotypic antibodies together with low affinity and avidity of autoantibodies in aged individuals is probably more important in this regulation (Doria G 1978; Arreaza EE 1993; Zhao KS 1995).

Although high incidence of autoimmune diseases is noted in the elderly, many of these diseases have started earlier in life and their presence in aged individuals simply reflects the fact that nowadays medicine allows affected individuals reaching the age ≥ 65 years. It has to be stressed that the characteristics of late-onset autoimmune diseases, *i.e.* diseases starting mainly in the elderly, differs from those starting earlier. For example, pernicious anaemia, Sjögren syndrome, myasthenia gravis are relatively slowly progressing as compared to a dramatic course of, occurring mainly in children, diabetes mellitus type I. Taking into account that the pressure of autoimmune phenomena in the elderly is thought to be much higher than that in the young, it seems that immunoregulatory mechanisms in aged individuals might be surprisingly well-preserved. Is it due to the accumulation of Treg cells? Obviously, Treg cells are only a small piece of the puzzle.

2.6 Interventions—Vaccinations

Prophylaxis with vaccines in the elderly is one of the most important medical interventions protecting from exacerbation of symptoms of various medical conditions which very often complicate infections at this age. The most advised for the elderly are anti-influenza and pneumococcal vaccines. As a leading goal of this form of therapy is to transform naïve lymphocytes into specific memory/effector cells, immune alterations associated with immunosenescence make it more difficult than in the young. The accumulation of Treg cells should be considered as a one of such harmful alterations. Treg cells were found to limit postimmunization effector and memory cell numbers (Toka FN 2004; Belkaid Y 2005). Consequently, depletion of Treg cells in animal model resulted in improved immune responses to variety of vaccines (Moore AC 2005). It is clinically relevant in geriatrics as Treg cells accumulate the most in frail elderly, that is, patients at the highest risk of complications, if the vaccination did not protect them from infection (Trzonkowski P 2006). Both serological and cellular protection achieved after anti-influenza vaccination was found to be the lowest in such individuals (Trzonkowski P 2003). The association, at least in case of cellular response, was not a co-incidence but proved interrelation as in vitro studies revealed that the addition of Treg cells to the cultures of CTL or NK cells resulted in the suppression of responses to the vaccine antigens. Cell-to-cell interactions were revealed

as the leading mechanism of this suppression (Trzonkowski P 2004). However, in some experimental types of vaccinations, IL10 secreted by Treg cells was the master regulator of immunization efficiency (Stober CB 2005). It has to be mentioned at this point that surprisingly low efficiency of vaccines against some pathogens, mainly parasites, is highly attributed to the effect of immune evasion that involves Treg cells. Namely, parasites protects themselves utilizing host Treg cells that suppress the action of the host effector mechanisms. Such situation during immunization against parasites results in low clinical effectiveness of anti-parasite vaccines (Belkaid Y 2005). Some indirect effects might be also very much relevant to the final outcome of the vaccination. For example, Treg cells may limit production of specific antibodies via suppression of CD4+ T helper cells cooperating with B cells during antigen encounter. As a result, the titer of specific protective antibodies after vaccination is low in patients characterized by high number of Treg cells, that is, mainly frail elderly (Trzonkowski P 2003). Also the fact, that Treg cells are consumers of IL2 might additionally decrease effectiveness of the immunization as this cytokine is necessary to generate protective post-immunization immune memory (Effros RB 1983; Provinciali M 1994). Again, Treg cells make the deficit of IL2 more severe in the group of patients characterized already by the lowest levels of this cytokine, *i.e.* in frail elderly (Trzonkowski P 2003). High number of Treg cells prior to the vaccination is not the only obstacle for efficient responses. It has been shown that immunization on its own, due to the challenge with administered vaccine peptides, generates vaccine-specific Treg cells which may additionally decrease immunization efficiency (Bauer T 2007). Specificity of Treg cells seems to be a key point in obtaining good post-immunization responses. For example, low response of CTL after immunization with immunodominant peptide of *Herpes simplex virus* was attributed to antigen-specific Treg cells. Namely, Treg cells isolated from mice chronically infected with *Herpes simplex virus* were much more potent in the suppression of *anti-herpes* CTL responses than Treg cells obtained from healthy mice (Suvas S 2003). In contrary, there are experimental data from mice which proves that very potent graft-specific Treg cells that keep operational tolerance to transplanted organs in recipient animals are not the obstacle in efficient cytotoxic responses to the challenge with influenza virus antigens as these antigens are different from those expressed by the graft (Bushell A 2005).

Interestingly, in some models Treg cells were required to receive efficient postimmunization responses. For example, depletion of Treg cells was associated with poor antibody responses to the vaccination and subsequent challenge with *Borrelia burgdorferi* (Nardelli DT 2006). Another interesting mode of regulation, in which regulatory T-cells are necessary to maintain post-immunization immune memory, is provided by the hypothesis of anti-idiotypic T-cells. This theory states that after a challenge with a given peptide, CD4+ T-cells create a network of idiotypic /anti-idiotypic T-cells (to some extent it is similar to Jerne's idiotypic network of antibodies; Nayak R 2001). According to this theory, antiidiotypic T-cells were in fact antigen-specific Treg cells, which presence was necessary to maintain long-term immune memory within both B and T subsets (Nayak R 2001; Lal G 2006). Some dysfunctionalities in this complex network over years of life might have been responsible for insufficient responses to vaccines in the elderly.

2.7 Interventions—Transplantation

Modern medicine has reached the point when the barrier of age is less important and even high-level invasive medical procedures are considered to be applied in the elderly. It is the most obvious in transplantation, which significance in geriatrics increases parallel with increasing number of elderly patients that received transplanted cells or organs. It is not a long time since modern drugs harnessed the major problem in transplantation, that is, incidence of acute rejections. Yet, their action is associated with many severe adverse effects which limit their use. It is of special importance in the elderly, in whom their administration may additionally deteriorate existing medical conditions or, in some cases, it is precluded due to insufficiency of organs taking part in their metabolism. Thus, dose reduction, application of novel less toxic drugs or tolerance induction strategies are one of the primary goals of nowadays transplantation. Fortunately, immunity compromised with age can be considered as an ally in these strategies. There are number of organs which have been reported to be better tolerated in the elderly as compared to the young after transplantation. Lower incidence of acute rejections in the elderly was reported in kidney, liver, heart, lung and corneal transplantations (Renlund DG 1987; Snell GI 1993; Vail A 1997; Zetterman RK 1998; Bradley BA 2000). While the majority of these studies are based on limited number of patients, renal transplantations can be analyzed with great statistical accurateness due to the widespread of this procedure. The analysis of around 80, 000 cases from the United Network of Organ Sharing (transplant registry in the US) fully confirmed that the level of acute rejections is lower in the elderly and the dose of immunosuppressive drugs in the elderly might be reduced (Bradley BA 2001, 2002). The need for reduced immunosuppression protocols in the elderly is urgent as the majority of posttransplant deaths at this age is associated with exacerbated circulatory diseases, tumors and infections, which are clear adverse effects of overimmunosuppression (Bradley BA 2001; Dębska-Ślizień A 2007). No doubt, accumulation of Treg cells with age may contribute to the deterioration of immunity and better transplantation outcomes in the elderly. Importantly, the action and number of Treg cells is modified with the use of particular immunosuppressive drugs which might have implications in establishing of immunosuppression protocols. For example glicocorticosteroids and mTOR inhibitors have been found to increase the number and function of Treg cells (Fattorossi A 2005; Game DS 2005), while calcineurin inhibitors depressed the activity of those cells (Zeiser R 2006). Of note, mTOR inhibitors are superior above other immunosuppressants as they have less adverse effects. This feature can make mTOR inhibitors a "drug-of-choice" in the elderly (Halloran PF 2004). Also the dose of strong and toxic calcineurin inhibitors can be reduced in the elderly as it was found that aged T-cells activated with alloantigens are less resistant to these drugs than T-cells from young recipients (Bradley BA 2001b). Surprisingly, the incidence of chronic rejections, currently known as chronic allograft nephropathy (CAN), is higher in the elderly than in the young. The most widely described cause of CAN is oversecretion of TGFβ (Suthanthiran M 1997). As already mentioned, the level of this cytokine is increased in the elderly but, apart from lymphocytes, it is secreted by a variety

Table 1 Regulatory subsets in humans

Subset	Phenotype	Mechanism	Origin
Naturally occurring CD4+ T regulatory cells	CD3+CD4+CD25^{high}FoxP3+ CD127-*	Cell-to-cell contact	Thymus
Adaptive CD4+ T regulatory cells	CD3+CD4+FoxP3+	Cell-to-cell contact	Conversion from nonregulatory CD4+ T-cells at the periphery
CD4+ Tr1 cells	CD3+CD4+IL10+ROG+	via IL10	Conversion from non-regulatory (usually naïve) CD4+ T-cells at the periphery
CD4+ Th3 cells	CD3+CD4+TGFβ+	via TGFβ and sometimes IL10	Conversion from non-regulatory (usually naïve) CD4+ T-cells at the periphery
CD28-CD8+ type 1 T suppressor cells	CD3+CD28-CD8+	Cell-to-cell contact, DC-dependent	Terminally differentiated CD8+ T-cells (generated in vitro by multiple rounds of stimulation with APC)
CD28-CD8+ type 2 T suppressor cells	CD3+CD28-CD8+IL6+IFNγ+	via soluble factors, IL6 and IFNγ required	Terminally differentiated CD8+ T-cells (generated in vitro in 1-week coculture with monocytes, GM-CSF and, IL2)
CD28-CD8+ type 3 T suppressor cells	CD3+CD28-CD8+IL10+	via IL10	Conversion of naïve CD8+ T-cells by IL10-producing plasmacytoid DC
NKT cells	'Classical' NKT: CD56+ CD3+TCRαβ(Vα24i) +'Nonclassical' NKT: CD56+CD3+TCRγd(Vγ9/ Vd2)+	via IL10, IL13	Thymus
Immature dendritic cells	Lin^{neg}HLA-DR+CD80^{low} CD86^{low} CD83^{low}	Cell-to-cell contact, IL10 and tryptophan deprivation (IDO)	From myeloid and lymphoid precursors
Plasmacytoid dendritic Cells	Lin^{neg}HLA-DR+CD11c^{low}CD123^{high}	Cell-to-cell contact, IL10 and tryptophan deprivation (IDO)	From lymphoid precursors
Cytokine-modulated mature dendritic cells	Lin^{neg}HLA-DR+CD80^{high} CD86^{high}CD83^{high}	IL10, TGFβ, TNFα, GM-CSF, G-CSF, M-CSF, VIP, IL21, thymic stromal lymphopoietin	From immature DC

* Other markers suggested but also expressed on other subsets: GITR+, CTLA-4+, neuropilin1+, CD45RB-, CD103+, CD62L+, CD54+, CD122+, CD134+, CD137+

of nonimmune cells. In transplanted kidney affected by CAN, TGFβ secreted by fibroblasts is suspected to be responsible for fibrosis, medial hyperplasia and therefore vessel narrowing. It is very much possible that proinflammatory activity in the elderly may contribute to CAN as proinflammatory cytokines directly stimulate production of TGFβ and activate mononuclear cells facilitating their trafficking and infiltration of the graft (Bradley BA 2002). Not to mention, that synergistic action of TGFβ and proinflammatory cytokines results in the generation of highly inflammatory Th17 cells. However, it has to be highlighted that pathogenesis of CAN is complex and consists of plenty, also non-immune, factors.

2.8 Interventions—Perspectives

Adoptive transfer or depletion of Treg cells is recognized as a manoeuvre suppressing or improving immune response, respectively. Bearing in mind that one of the major features of immune risk phenotype is a low level of dysfunctional CD4+ T-cells, intervention affecting CD4+ T-cells might be of interest in geriatrics. In theory, depletion of Treg cells can be specifically obtained using anti-CD25 antibody, the drug commonly used during allogeneic transplantations in humans. Although initially confirmed, the depletion was subsequently denied by other reports (Kreijveld E 2007). It was reported in some oncological studies that the administration of the antibody improved anti-tumor responses but did not kill but rather blocked the activity of Treg cells (Fecci PE 2006). On the other hand, the use of other antibodies in humans, such as anti-CD3, anti-CD52, anti-lymphocyte globulin preparations, was reported to induce different subsets of Treg cells (Belghith M 2003; Ciancio G 2005; Lopez M 2006). Currently, a lot of effort has been put into attempts of ex vivo large-scale generation of Treg cells which might be subsequently used as immunosuppressive medication (Tang Q 2006). Apparently, in the light of the fact that Treg cells accumulate in aged subjects, these attempts seem to be irrelevant for the elderly. Nevertheless, aged population is a substantial consumer of various immunosuppressive drugs and therapy with Treg cells is thought to be substantially less toxic alternative to those drugs.

3 Other Regulatory Subsets

3.1 Dendritic Cells

Although CD4+ T-cells are robust in their regulatory abilities, it is not the only subset having such potential. It is not surprising that DC are also considered as they are often the first sensors of pathogen pattern. Their action directs all subsequent responses of the immune system. While mature DC trigger mainly robust effector responses, immature DC have the capability of immunosuppression in

order to protect self tissue against uncontrolled effector activity of immune system. DC might be of importance as they seem to be relatively slightly affected by age (Agrawal A 2007). Some known age-dependent alterations in DC functioning, such as low expression of MHC receptors and costimulatory molecules (Shurin MR 2007), make the phenotype of aged DC close to immature tolerogenic DC. Namely, it is widely known that immature DC, that is, DC with low expression of MHC and costimulatory molecules, are capable of induction of anergy in effector T-cells and transition of naïve T-cells to adaptive subsets of Treg cells (Steinbrink K 1997; Steinbrink K 1999; Jonuleit H 2000; Vigoroux S 2004). Immature DC are so effective in this process as they have become a laboratory tool in expansion of Treg cells for therapeutic purposes (Yamazaki S 2006). The mechanism of action of tolerogenic DC is associated with release of IL10 (by DC and adaptive Treg cells stimulated by DC) and expression of indoleamine 2,3-dioxygenase (IDO; Steinbrink K 1999; Munn DH 2002). The latter mechanism is very intriguing as increased expression of this enzyme in immune cells of aged individuals was described as predictive for mortality (Pertovaara M 2006). As lymphocytes require tryptophan for their proper functioning, its deprivation triggered by IDO is recognized as ``immunosuppression by starvation of immune system'' (Mellor AL 1999). In addition, IDO in DC metabolises tryptophan to kynurenines and these products suppress T-cells. To a great extent, anergy of T-cells in such environment is dependent on upregulation of GCN2 kinase in T-cells and induction of adaptive Treg cells (Munn DH 2002; Mellor AL 2003). The activity of the enzyme was found to be increased in late-onset autoimmune diseases and chronic infections (Mellor AL 1999; Pertovaara M 2005). Interestingly, the activity of IDO, including the isoform expressed in immune cells, is associated with serotonin deficit in depression (Cubala WJ 2006). There are assumptions that the enzyme might be an important link between chronic stress, inflammation and neurohormonal alterations in this disease (Muller N 2007). As depression is one of the most important medical conditions in the elderly and serotonin deficit is the target for a very potent group of antidepressive drugs (SSRI), the research upon IDO will for sure find its continuation in the elderly. Recently, a growing attention in the field of immune regulation has been given specifically to immature plasmacytoid DC (PDC or DC2) which were found to be extremely powerful regulators of immune responses. Utilizing IDO-related mechanisms, immature PDC significantly reduce antigen presentation, which leads to immunosuppression (Munn DH 2004). Induction of anergy of CD4+ T-cells by immature PDC occurs in direct cell-to-cell interaction between MHC and TCR receptor which prevents from upregulation of CD40L, and possibly other costimulatory molecules, on T-cells (Kuwana M 2001). Particular relevance of these cells for the clinic comes from the fact that the presence of immature PDC promotes vigorous progression of tumor growth and significantly decreases efficiency of anti-tumor vaccines (Munn DH 2004; Shurin MR 2007). While there is single study that reported no difference in the activity of PDC between young and adult mice (Dakic A 2004), there is still no convincing data on the activity of PDC in aged humans (Shurin MR 2007). It has to be mentioned that mature DC, in some specific conditions, are also capable of immunosuppression. It is mainly due to the action of various cytokines, such as

IL10, TGFβ, TNFα, GM-CSF, G-CSF, M-CSF, VIP, IL21 and, thymic stromal lymphopoietin, that modulate activity of mature DC (Rutella S 2006).

3.2 CD28− CD8+ T-Cells

CD28-CD8+ T-cells, described elsewhere in this book as a substantial burden for immunity in ageing, have some regulatory abilities when cooperate with other immune subsets. Because of that they are often described as T suppressor cells (Ts). Thus, anergy of these cells should be also evaluated in the context of regulation of particular immune responses. First of all, CD28-CD8+ Ts cells are heterogeneous with at least three subsets distinguished already (Filaci G 2002). Ts type 1 cells trigger anergy of CD4+ T effector cells through interaction with DC presenting specific antigens to these effectors (Liu Z 1998). As such, the inhibition is MHC-restricted. Anergy occurs due to the inhibition of expression of CD40 receptor on the surface of DC, which further prevents from upregulation of B7 molecules on DC. Ts type 1 cells also upregulate expression of the immunoglobulin-like transcripts ILT3 and ILT4 on DC (Chang CC 2002). The expression of these transcripts upon stimulation with Ts type 1 cells not only is responsible for anergy of CD4+ T effector cells but also for promotion of adaptive Treg cells (Suciu-Foca N 2005). Ts type 2 cells were generated from CD8+ T-cells in vitro in the presence of monocytes, exogenous IL2 and GM-CSF (Balashov KE 1995). These cells are capable of suppression of cytotoxic cells via secreted cytokines in MHC unrestricted way (Filaci G 2002). Interestingly, IL6 and IFNγ secreted by Ts type 2 cells were indispensable in this mode of suppression (Filaci G 2001). Finally, Ts type 3 cells can be generated by the stimulation of naïve CD8+ T-cells with IL10-producing PDC. Ts type 3 cells acquires then the ability to secrete IL10 on their own and suppress other naïve, but not effector, CD8+ T-cells. IL10 secretion, rather than downregulation of CD28 receptor, is the characteristic feature of Ts type 3 cells. Despite IL10-dependent mode of action, the inhibitory effects appear to be antigen-specific and limited to the antigens presented initially by PDC to Ts type 3 cells (Gilliet M 2002).

Like in the case of many other elements of immune response, generation of CD28-CD8+ T-cells might be considered profitable during particular infections as these cells control effector cells and prevent from damage of self tissues. On the other hand, frequent infections, accumulation of infectious episodes throughout life or chronic form of infections may result in continuous generation of CD28-CD8+ T-cells which skews effector/ suppression balance during aging towards suppression (Pawelec G 2005). Important way of escape from this age-dependent dysregulation, a kind of "rescue circuit of regulation," might be acquired expression of KIR receptors on CD28-CD8+ T-cells (Abedin S 2005). Unlike the level of intrinsic NKT cells, the level of NK-like T-cells expressing KIR receptors is increased in aged individuals (Tarazona R 2000; Peralbo E 2007). The expression of KIR receptors is not a constant feature of CD28-CD8+ T-cells and becomes evident at late phase of their differentiation (Arlettaz L 2004). Diversity and function

of KIR receptors implies that the expression of some of them make CD28-CD8+ T-cells tolerant towards self antigens, while the others are capable of triggering their cytotoxic response. Thus, it is probable that the expression of different sets of KIR receptors might be responsible for fine tuning of the function of CD28-CD8+ T-cells (Abedin S 2005).

3.3 NKT-Cells

Intrinsic NKT-cells are another subset with regulatory abilities. NKT-cells merge the characteristics of T-cells and NK-cells but the facts that the repertoire of their TCR is restricted (invariant Vα and limited diversity of Vβ) and they recognize very limited range of glycolipids, via CD1d on APC cells classically, place them on the border between acquired and innate immunity (Biron CA 2001; Kinjo Y 2005). Their proportion in peripheral blood is small and reaches not more than 2–3% of T-cells. In peripheral tissues they preferentially migrate to the bone marrow and liver, where they constitute 10–20% and 30–40% of T-cells, respectively (Emoto M 2003). The most classically, NKT cells are generated in the thymus and traffic mainly to the liver (Abo T 2000). The number of NKT-cells was found to be increased in aged mice but diminished in the general population of the elderly humans with exception of very old subjects. It might be important for aged immune system that the liver can serve as a site of extrathymic development of NKT-cells which results in increased number of those cells in centenarians (Watanabe H 1996; Miyaji C 2000). Regardless of number discrepancies, it is altered function of NKT-cells that influences substantially the activity of immune system (DelaRosa O 2002; Faunce DE 2005; Peralbo E 2007). On the one hand, NKT-cells were found to control autoimmune diseases, such as diabetes mellitus type I, rheumatoid arthritis, inflammatory bowel disease, systemic sclerosis (Sumida T 1995; Hong S 2001; Lee PT 2002; van Kaer L 2005) and promote tolerance to transplanted organs (Jiang X 2005, 2007), but on the other hand, they significantly improve anti-tumor responses and potentialize efficiency of vaccines (Cui J 1997). These ambiguous results might be explained by the mechanisms of their action, which suggest their regulatory activity (Kronenberg M 2005). When stimulated, they produce both Th1 and Th2 cytokines. Production of Th1 cytokines, mainly IFNγ, is responsible for augmented anti-tumor and viral responses via stimulated NK cells (Cui J 1997), while Th2 cytokines, mainly IL4 and IL10, are responsible for NKT-mediated suppression (Kronenberg M 2005). Ageing is associated with decreased secretion of IFNγ by NKT cells, which is recognized as an important reason of deficits in antiviral and antitumor responses in the elderly (Miyaji C 2000; Mocchegiani E 2004). On the other hand, the secretion of Th2 cytokines, like IL10, remains unchanged or even increases with age (Faunce DE 2005). It seems to be a powerful regulatory mechanism as the secretion of IL10 by NKT cells was found to be a major inducer of tolerance to many allotransplants (Oh K 2005; Jiang X 2007). Moreover, increased suppressive activity of NKT cells stimulate secretion of IL10 by CD4+ and CD8+ T-cells and DC (Jiang X 2007;

Wahl C 2007). NKT cells were also found to suppress CD8+ T-cells via secreted IL13 (Terabe M 2000). However, the most convincing proof of the regulatory activity of NKT cells comes from the fact of reciprocal influence of NKT cells and Treg cells. Some NKT cells were found to secrete IL2 stimulating proliferation of Treg cells (Jiang S 2005). In several models, NKT cells were found to promote oral tolerance via induction of adaptive Treg cells which secreted IL10 and TGFβ (Roelofs-Haarhuis K 2004; Kim HJ 2006). Also the shift towards Th2 cytokines produced by gut-associated NKT cells was revealed to be associated with local increase in the number of Treg cells (Ronet C 2005). In contrary, Treg cells were found to suppress activity of NKT cells in cell-to-cell manner (Azuma T 2003). It is probably relevant in the clinic as the interference of Treg cells with NKT cells was proved to promote enhancement of some tumors (Nishikawa H 2003).

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B Cells

Transcription Factors in Mature B-Cells During Aging

Daniela Frasca, Richard L. Riley and Bonnie B. Blomberg

Contents

Abbreviations

AID	activation-induced cytidine deaminase
ARE	adenylate/uridylate-rich elements
BAFF	B-cell-activating factor
BCMA	B-cell maturation antigen
bHLH	basic helix loop helix
BSAP	B-cell lineage-specific activator protein
CSR	class switch recombination
EMSA	electrophoretic mobility shift assay
HEB	E-box binding protein
Ig	Immunoglobulin
MZ	marginal zone
NF-κB	nuclear factor-κB
RAG	recombination activating enzyme
RT-PCR	reverse transcription-polymerase chain reaction
SL	surrogate light (chain)
TACI	transmembrane activator and CAML interactor
TdT	deoxynucleotidyl transferase
TD	thymus-dependent
TI	thymus-independent
TTP	tristetraprolin
UTR	untranslated region

D. Frasca (✉) · R. L. Riley · B. B. Blomberg
Department of Microbiology and Immunology
University of Miami Miller School of Medicine
P.O. Box 016960 (R-138)
Miami, FL 33101, USA
Tel.: 305-243 6225
Fax: 305-243 4623
E-mail: dfrasca@med.miami.edu

D. Frasca
Graduate School of Cell Biology and Development
University of Rome La Sapienza
Rome, Italy

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Abstract: The purpose of this chapter is to give an overview of the age-related changes in the expression and function of the major transcription factors regulating mature B-cells. We also summarize our recent work and show that the age-related defects in Ig class switch are directly related to the decrease in the transcription factor E47 which controls the expression of AID, needed for CSR. The age-associated effects on the expression and function of the transcription factors NF-κ B and Pax-5 are also described. Blimp-1 seems not to be modified by aging. For other transcription factors relevant for mature B-cell functions, such and IRF4 and Notch2, no effects of aging have been reported so far. The defects presented herein for aged B-cells should allow the discovery of mechanisms to improve humoral immune responses in both humans and mice in the near future.

1 E Proteins

Class I basic helix loop helix (bHLH) proteins, also known as E proteins, were first identified based on their ability to bind with relatively high affinity to the palindromic DNA sequence CANNTG, referred to as an E-box site (Ephrussi et al. 1985; Henthorn et al. 1990; Quong et al. 2002), found in the promoter and enhancer regions of many B lineage-specific genes, such as the enhancers in the immunoglobulin (Ig) loci and the promoters of mb-1, λ5 and RAG-1 (Quong et al. 2002). The E protein family includes E12, E47, HeLa E-box binding protein (HEB), and E2-2, in vertebrates, and the Drosophila gene product, daughterless (Massari and Murre 2000). E12 and E47, arising through differential splicing of the exon encoding for the HLH domain of the *E2A* gene (Murre et al. 1989), regulate a plethora of processes involved in B-cell commitment and differentiation. In particular, they initiate Ig rearrangements; and regulate the expression of the surrogate light (SL) chain, the recombination activating enzymes RAG-1 and RAG-2, the enzyme terminal deoxynucleotidyl transferase (TdT), the IL-7Rα chain, which together with the common γ chain (γc) comprises the high affinity IL-7 receptor (IL-7R), and the genes encoding the signal transduction molecules Igα (mb-1) and Igβ (B29; Schlissel et al. 1991; Sigvardsson et al. 1997; Massari and Murre 2000; Kee et al. 2002). E2A also induces the expression of EBF, which acts in synergy with E2A to promote SL chain transcription.

In B lymphocytes, the active DNA-binding complex consists of E47 homodimer, as opposed to E12/E12 or E12/E47 complexes, whereas in the bone marrow pro-B/early pre-B cells the predominant form is E12/E47 (Frasca et al. 2003). The formation and the function of the homodimer or heterodimer depend on the balance between the *E2A*-encoded proteins, other class I bHLH proteins (HEB and E2-2) and the E protein inhibitory proteins, Id 1-4, which lack the DNA-binding domain and function as dominant negative inhibitors of E proteins (Rivera and Murre 2001). The paradigm of HLH function is that an ubiquitously expressed class I bHLH protein dimerizes with a tissue-specific class II factor, such as MyoD (skeletal muscle) or NeuroD (neurons), to regulate cell-specific gene transcription. E2A-deficient

mice display a complete block in B lineage development at a very early stage prior to the onset of IgH DJ rearrangement, whereas myeloid development is normal. Transgenic introduction of either E47 or E12 restores B lymphopoiesis in E2A-deficient mice, although E47 promotes pre-B cell differentiation more effectively, likely because it has a higher DNA-binding affinity than does E12 (Shen and Kadesch 1995). Mice expressing a transgene for Id proteins, the inhibitors of E protein activity, have a phenotype similar to the $E2A^{-/-}$ mice (Quong et al. 2002). These mice display the same block in B-cell development, and its severity is dependent on the level of expression of the transgene.

E2A activity is necessary for class switch recombination (CSR; Quong et al. 1999; Sugai et al. 2003), as the E47 transcription factor has been shown to be important in transcriptional regulation of *Aicda*, the gene encoding the activation-induced cytidine deaminase (AID; Sayegh et al. 2003), the enzyme responsible for breaking the DNA in the switch regions, the first step in the CSR process. Briefly, it has been shown that ectopic expression of Id3 in splenic activated B-cells inhibits CSR (Quong et al. 1999) because of reduced AID transcription and overexpression of E47 can directly induce *Aicda* gene expression both in a B-cell line and in splenic B-cells activated in vitro (Sayegh et al. 2003). A cis-regulatory element (E-box) in the *Aicda* locus has been identified and shown to be activated by E-proteins. Ectopic expression of AID in splenic activated B-cells retrovirally transduced with Id3 only partially rescues the ability of these cells to undergo CSR. The Authors concluded that the efficient induction of *Aicda* expression is dependent on E-proteins, but also suggest that E-proteins have roles in CSR in addition to their induction of *Aicda* expression. However, the level of restoration of AID was not complete in these experiments and therefore an alternative interpretation of these results would be that optimal E47, which would induce optimal AID, would itself completely restore CSR. Our data showing no decrease in germline μ transcripts in old or in $E2A^{+/-}$ B-cells support this hypothesis (Frasca et al. 2004a).

In senescent mice, we have previously shown that in vitro stimulated splenic B-cells are deficient in production of multiple class switch isotypes (IgG1, G2a, G3, and E), and CSR (Frasca et al. 2004a, b). This occurs concomitant with decreased induction of E47 and AID. The reduced CSR observed in old splenic activated B-cells is not the consequence of defective B-cell proliferation, as B-cells from old mice can be effectively activated in vitro, but their capacity to undergo CSR is impaired. Our results are in line with the findings that expression of the receptors for CD40, and IL-4 are unaffected by aging in mice and humans, as already reported (Whisler et al. 1991; Song et al. 1997; Zheng et al. 1997; Bergler et al. 1999). Although it is known that there are defects in T as well as B-cells during aging, our studies indicate that an intrinsic B-cell defect may not be able to be rescued by modifying/enhancing T-cell activity alone by itself in aged individuals. Both DNA-binding (EMSA) and expression (Western blot) of E47 are decreased in stimulated splenic B-cells from old mice. We have previously shown (Frasca et al. 2003) that the endogenous E47 DNA-binding is low, and importantly, twofold lower than that in unstimulated young spleen cells in the majority of aged mice individually tested (65%). Activation of B-cells up-regulates E47 DNA binding in

young and to a significantly lower extent in old mice. Therefore, both basal and activated levels of E47 are decreased in splenic B-cells in aged mice. These findings suggest that the down-regulation of this transcriptional regulator may help explain not only decreased CSR in activated splenic B-cells from old mice, but also age-related changes in affinity maturation and SHM affecting the quality of the Ab response. Other results from our laboratory showing that CSR is perturbed in $E2A^{+/-}$ mice further support the important role of this transcription factor in the generation of Abs with different isotypes (Frasca et al. 2004a).

In order to determine a mechanism for the age-related decrease in the amounts of E47 protein in nuclear extracts, we found that E47 mRNA levels were decreased in stimulated splenic B-cells from old as compared with young mice. RNA stability assays showed that the rate of E47 mRNA decay was accelerated in stimulated splenic B-cells from old mice, but E47 protein degradation rates were comparable in young versus aged B-cells, indicating that the regulation of E47 expression in activated splenic B-cells occurs primarily by mRNA stability (Frasca et al. 2005b, 2007b). In contrast with splenic activated B-cells, E47 mRNA expression is comparable in bone marrow-derived IL-7-expanded pro-B/early pre-B cells from young and old mice (Van der Put et al. 2004). Thus, the reduced expression and DNA-binding of the E12/E47 transcription factor in aged B-cell precursors is not transcriptionally regulated, but is due to reduced protein stability (Van der Put et al. 2004; King et al. 2007) mediated presumably via the ubiquitin–proteasome pathway (Kho et al. 1997; Huggins et al. 1999). This instability is largely due to PEST (proline, glutamic acid, serine, threonine) residues common to degradation domains (Huang et al. 1998).

The stability of labile mRNA may be controlled by signal transduction cascades, where the final product of the cascade phosphorylates a protein which interacts with adenylate/uridylate-rich elements (ARE) in the 3′ untranslated region (UTR) of mRNA and modifies its stability (Chen et al. 1995; Bevilacqua et al. 2003). ARE sequences have been found in the 3′-untranslated region (UTR) of many mRNAs, including those for transcription factors. ARE motifs have been previously classified into at least three categories based in part upon the distribution of AUUUA pentamers. Class I AREs, found in early response gene mRNAs like c-fos and c-myc, contain multiple isolated AUUUA motifs; class II AREs, found exclusively in cytokine mRNAs, contain two or more overlapping copies of the AUUUA motif; class III AREs contain no AUUUA motifs but generally contain U-rich or AU-rich regions and possibly other unknown features for their destabilizing function. The E47 mRNA is a class I/III mRNA, because it has one AUUUA sequence and multiple AU/U-rich regions. At least part of the decreased stability of E47 mRNA seen in aged B-cells is mediated by proteins. We have found that tristetraprolin (TTP), a physiological regulator of mRNA expression and stability, is involved in the degradation of the E47 mRNA. Because many studies have shown TTP expression and function in macrophages, monocytes, mast cells and T-cells, but little is known about the expression and function of TTP in primary B-cells, we have investigated TTP mRNA and protein expression in splenic B-cells from young and old mice. Our recently published results (Frasca et al. 2007b) show that TTP mRNA and protein levels are higher in stimulated splenic B-cells

from old as compared with young mice. TTP has been described to be directly phosphorylated by p38 MAPK in macrophages (Carballo et al. 2001; Chrestensen et al. 2004; Cao et al. 2006). We show that inhibition of the p38 MAPK signaling pathway significantly reduces TTP protein expression in B-cells. Old B-cells in response to LPS make less phospho-p38 MAPK (Frasca et al. 2007b) and therefore, as would be expected, make less phospho-TTP. This leads to an increase in the amount of TTP bound to the 3′-UTRs, and therefore decrease mRNA stability (of E47) in old B-cells. Our studies demonstrate for the first time that TTP is regulated in activated B-cells during aging, that TTP is involved in the degradation of the E47 mRNA, and show the molecular mechanism for the decreased expression of E47, AID and CSR in aged B-cells.

2 NF-κB

The transcription factor, nuclear factor-κ B (NF-κB), has also been shown to be important for Ig class switch (Snapper et al. 1996). NF-κB has been shown to be strongly activated by anti-CD40/IL-4, but not by anti-CD40 or IL-4 stimulation alone in splenic B-cells and to be involved in CSR to IgG1/IgE in both humans (Jeppson et al. 1998) and mice (Tinnell et al. 1998; Pioli et al. 1999; Kaku et al. 2002). It has also been shown to be the key transcription factor in mouse or human B-cells undergoing CSR in response to BAFF, the B-cell-activating factor, also called BLyS, TALL-1, THANK, ZTNF4 or TNF13B (Litinskiy et al. 2002; Castigli et al. 2005; Yamada et al. 2005).

We have recently investigated the ability of BAFF/IL-4, as compared to anti-CD40/IL-4, to induce CSR to γ_1 in splenic B-cells from young and old mice (Frasca et al. 2007a). We found that anti-CD40/IL-4 is a better CSR stimulus than BAFF/IL-4 in young B-cells, as measured by RT-PCR of postswitch transcripts and flow cytometry. CSR is reduced in old B-cells with both stimuli, but the suboptimal CSR seen in young mice to BAFF/IL-4 shows less reduction in the old B-cells. AID and γ_1PSTs are significantly reduced in old B-cells stimulated with anti-CD40/IL-4, and less reduced with BAFF/IL-4 stimulus. BAFF receptor mRNA expression (BAFF-R, TACI, and BCMA) is not affected by aging. The age-related decrease in CSR induced by anti-CD40/IL-4 is primarily associated with a decrease in E47, whereas the less affected response to BAFF/IL-4 is associated with decreases in both E47 and NF-κB. Therefore, NF-κB is not involved in the decreased response of old B-cells to anti-CD40/IL-4. These differences in B-cell responses to CD40/IL-4 and BAFF/IL-4 may help to explain at least a partial maintenance of TI (more BAFF/IL-4-dependent) versus TD responses in senescent mice (Smith 1976; Weksler et al. 1978).

The mechanisms by which NF-κB controls CSR are known only in part. Recent results show that signals delivered via CD40 that activate NF-κB synergize with signals delivered via the IL-4 receptor that activate Stat-6 to induce optimal AID gene expression (Dedeoglu et al. 2004). The importance of Stat-6 and NF-κB in induction of AID expression by IL-4 and CD40 was demonstrated in studies of Stat-6$^{-/-}$ and p50$^{-/-}$ mice. However, in this study (Dedeoglu et al. 2004) the ability of

CD40 ligation to induce AID expression and to synergize with IL-4 in AID induction in B-cells was only partially impaired in $p50^{-/-}$ mice, suggesting that NF-κB is only one of the transcription factors involved in inducing AID expression and CSR in B-cells. Our studies show that the defect in aging seen in CSR is due primarily to E47 and not to NF-κB (Frasca et al. 2007a).

3 Pax-5 (BSAP)

Pax-5, also called B-cell lineage-specific activator protein (BSAP), is critical for B-cell lineage commitment, B-cell development and CSR in GC B-cells, but it is not expressed in terminally differentiated B-cells (Max et al. 1995; Nutt et al. 1998, 1999; Linn et al. 2002; Gonda et al. 2003). B-cell-specific target genes for Pax-5 are λ5, CD19, mb-1, blk, RAG-2, J-chain, and IgH genes (Kozmick et al. 1992; Neurath et al. 1994; Zwollo et al. 1994; Michaelson et al. 1996; Lauring and Schlissel 1999). Binding sites for Pax-5 have been identified in the promoters of multiple genes as well as at multiple sites within the IgH locus (Neurath et al. 1994; Michaelson et al. 1996). Pax-5-dependent repression of X box binding protein-1 (XBP-1) is probably critical for inhibiting plasmacytic differentiation in the GC (Shaffer 2002). It has recently been demonstrated that a putative regulatory region in the *Aicda* gene contains both E2A- and Pax-5-binding sites, and the latter site is indispensable for AID gene expression (Gonda et al. 2003). Id proteins have been shown to interact with Pax-5, and inhibit its DNA-binding (Roberts et al. 2001; Gonda et al. 2003). E2A proteins have been described to regulate Pax-5 not directly but through its regulation of EBF (Kee and Murre 1998). Consistent with these observations is the finding that the Pax-5 promoter contains functional EBF binding sites (O'Riordan and Grosschedl 1999).

Pax-5 DNA-binding activity (for the active Pax-5a isoform) has been shown to be strongly reduced in resting splenic B-cells from aged mice, whereas protein levels did not change significantly (Anspach et al. 2001). Decreased Pax-5 binding activity is not the result of decreased levels of Pax-5 RNA transcripts or overall protein levels, as shown by RNase protection and Western blot analyses, suggesting a posttranslational mechanism affecting Pax-5 activity in aged B-cells, possibly involving its oxidation status (the oxidative form does not bind to DNA; Tell et al. 1998). Unlike E2A, Pax-5 is regulated posttranscriptionally in splenic B-cells. Preliminary results from our laboratory have shown that in splenic activated B-cells Pax-5 may also be regulated by mRNA stability (Landin, Frasca and Blomberg, work in progress).

4 Blimp-1

Blimp-1, encoded by the *prdm1* gene (Lin et al. 2003), is a transcriptional repressor which represses proliferation and induces maturation of B-cells into antibody-secreting plasma cells. It blocks the alternative GC B-cell fate by inhibiting Bcl-6,

Pax-5, BCR signaling, E2A, EBF, CSR, activation and homing to follicles (Lin et al. 2002; Shaffer et al. 2002; Johnson and Calame 2003, Calame et al. 2003). Blimp-1 has been detected in plasma cells, but not in early bone marrow B-cells, splenic memory B-cells in spleen, and GC B-cells (Tunyaplin et al. 2004).

As demonstrated by Han et al. (2003), there is a substantially higher number of antibody-secreting cells in the spleens of old mice than in the spleens of young mice. Therefore, we measured Blimp-1 mRNA expression in cultures of splenic B-cells from young and old mice activated for different times with LPS. We also determined the percentages of plasma cells ($CD138^{+}B220^{low}$) in cultures of LPS-stimulated B-cells from young and old mice. The expression of mRNA for Blimp-1 is induced by LPS and suppressed by IL-4 (Knodel et al. 2001). Our results (Frasca et al. 2004b) showed that Blimp-1 mRNA was undetectable in unstimulated B-cells, increased at days 2 and 3, reached the optimum levels at day 4 and then decreased at day 7 in both young and old mice. Blimp-1 mRNA expression was comparable in young and old splenic B-cells. These results again point to the main defect in aged stimulated B-cells being at the level of CSR, and not due to differentiation to antibody-secreting (plasma) cells.

5 IRF4

IRF4, also called Pip, LSIRF, ICSAT or MUM1 (Iida et al. 1997) is a member of the interferon-regulatory factor family of transcription factors characterized by a specific DNA-binding domain and by the ability to bind to regulatory elements in promoters of interferon-inducible genes. In the B lineage, IRF4 is expressed in immature B-cells in the bone marrow, is absent from proliferating centroblasts and then is re-expressed in plasma cells (Lu et al. 2003; Klein et al. 2006). IRF4, together with Blimp-1, is required for the generation of plasma cells, both transcription factors acting upstream of the transcription factor XBP-1 (Klein et al. 2006). No aging effects have been reported so far for IRF4.

6 Notch2

The Notch family of receptors plays an important role in the development of hematopoietic cells (Maillard et al. 2005). Notch1 regulates T-cell development, whereas Notch2 is preferentially expressed in mature B-cells (Saito et al. 2003). Conditionally targeted deletion of Notch2 results in a defect in marginal zone (MZ) B-cells and their precursors (Kuroda et al. 2003). Among Notch target genes, the expression level of Deltex1 is prominent in MZ cells and strictly dependent on that of Notch2, suggesting that Deltex1 may play a role in MZ cell differentiation. No aging effects have been reported so far for Notch2.

7 Conclusions

In conclusion, particular transcription factors have been shown to be decreased with age in activated murine B-cells, *e.g.* E47, or in resting B-cells, *e.g.* Pax-5. The stage of differentiation as well as activation of the cell types studied appears to be important as our data on E2A in murine bone marrow versus the spleen has shown decreases with age but using different molecular mechanisms (Frasca et al. 2005a; Riley 2005). Further studies should help to better determine the molecular mechanisms for these suboptimal expression of transcription factors, their molecular consequences, and provide avenues for correction of the immune deficiencies created by them.

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B-Cell Repertoire Changes in Mouse Models of Aging

Jean L. Scholz, William J. Quinn III and Michael P. Cancro

Contents

Abstract: Changes in the antibody repertoire are a well-established feature of immunosenescence. These reflect an aggregate of age-associated alterations in the generation, numbers, and proportions of B-cell subsets; as well as the homeostatic and selective processes governing them. A basic understanding of these relationships, coupled with integrated assessments of how they change with age, should reveal mechanisms underlying the immunosenescent phenotype. Mouse models provide powerful tools for these analyses, allowing controlled manipulation of key genetic, cellular, and microenvironmental factors. Here we summarize current understanding of how primary and antigen-experienced murine B-cell repertoires are established, as well as how they shift with age.

M. P. Cancro (✉)
Department of Pathology and Laboratory Medicine
University of Pennsylvania School of Medicine
36th and Hamilton Walk
Philadelphia, PA 19104-6082, USA
Tel.: 215 898 8067; Fax: 215 573 2350
E-mail: cancro@mail.med.upenn.edu

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1 Introduction

Immunosenescence, the progressive dysregulation of immune function with age, reflects a mosaic of genetic, epigenetic, and microenvironmental changes [10, 11, 34, 42, 44, 77, 101, 102, 125–127, 158]. This complexity confounds minimal explanations of the overall phenomenon, and underscores the need to exploit systems whereby defined factors can be deliberately manipulated. Accordingly, mouse model systems, which have been refined as immunologic experimental tools, should yield insights into the underlying mechanistic relationships.

Altered clonotype repertoires are a consistent feature of immunosenescence. This is anecdotally evident from the shifts in immune responsiveness, increased autoimmunity, and clonal expansions that accompany age; and is corroborated through empirical evidence in human and animal models. For example, both the frequency and clonotypic composition of hapten- and virus-specific primary B-cells change with age [76, 112–114, 136, 182, 184, 185]; and nearly all laboratory mouse strains display an age-associated appearance of autoantibodies [29, 30]. Most observations addressing these age-associated repertoire shifts are based on assessments at single time points. While these can *identify* repertoire changes, the underlying mechanisms resist interrogation via such static sampling approaches, because lymphocytes comprise multiple, dynamic populations under stringent selective and homeostatic controls.

Lymphocyte dynamics involve the continuous generation and corresponding loss of cells, such that relatively constant numbers are maintained. Thus, the stability of lymphocyte numbers disguises underlying and ongoing cellular and molecular processes. For example, commitment rates to the B lineage per se, as well as the entrance rates and lifespan of B-cells in different functional subsets, can vary. Further, these compartments not only play differing immunological roles, but also can interact with and impact one another's behavior. Finally, selective events based on B-cell receptor (BCR) specificity, innate ligand responsiveness, and homeostatic factors are superimposed on this dynamically changing landscape. Accordingly, effective interrogation of repertoire changes—including those associated with advancing age—requires simultaneous, longitudinal assessments of lymphocyte generation, homeostasis, and selection.

Indeed, the size, proportions, and dynamics of nearly all progenitor and mature B lineage subsets shift with age in the mouse, so overall changes in clonotype frequency and composition likely reflect the aggregate of these shifts. Understanding age-associated repertoire changes therefore requires an appreciation of the molecular and cellular mechanisms governing primary and antigen-experienced repertoires. Herein we review currently accepted notions about the identity and relationships of B lineage subsets and their progenitors, emphasizing the selective and homeostatic processes impacting repertoire composition. With this as background, age-associated changes in these parameters and their potential relationship to repertoire shifts in primary and antigen-experienced B-cell subsets are discussed. A schematic summary of these overall changes is provided in Fig. 1.

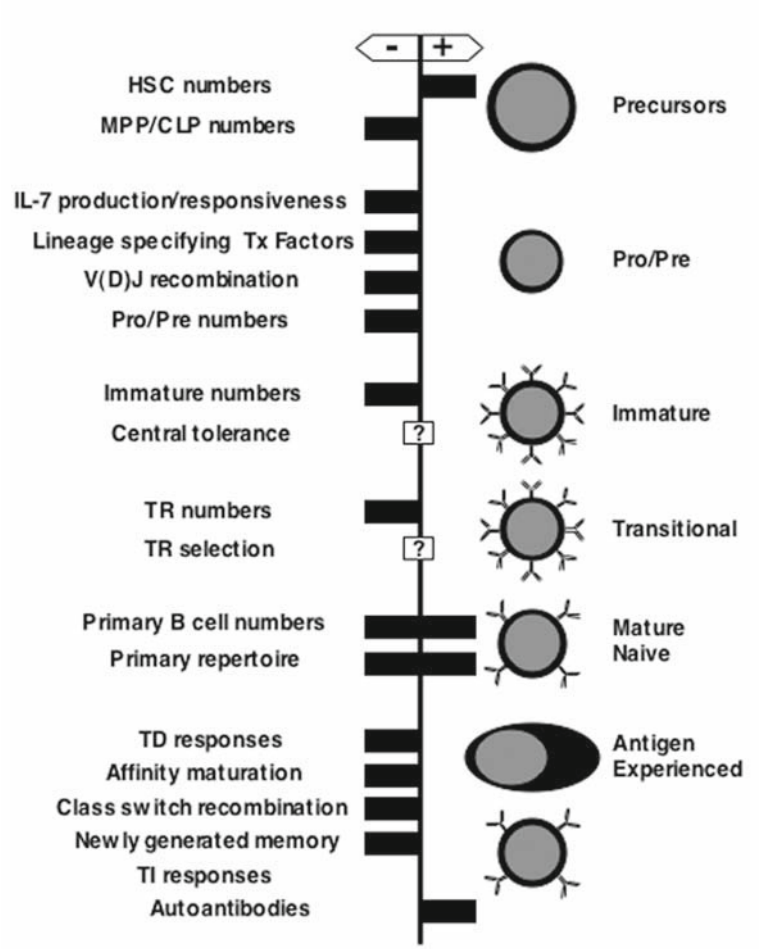

Fig. 1 Changes in B-cell traits with age. Major B-cell subsets are shown at right with characteristic traits and/or processes at left. Bars indicate changes in aged mice compared to young adults. Bars left-of-center denote age-associated reductions, while bars right-of-center indicate age-associated increases, of a characteristic. Disparate or mixed results are indicated as bi-directional bars, and currently unexplored issues are signified by a central question mark.

2 Primary Repertoire Development

2.1 Lineage Commitment and Developing Bone Marrow B-Cell Subsets

In adults, B-cells are generated in the bone marrow (BM), where pluripotent hematopoietic stem cells (HSCs) give rise to multipotent progenitor (MPPs) that initiate lymphoid-restricted gene expression. This yields common lymphoid pro-

genitors (CLPs) [65], a population enriched for B lineage specified precursors that subsequently become committed to the B lineage [2]. Several transcription factors and cytokine receptors comprise a regulatory network for B lineage specification and commitment [16, 66, 90, 106, 117, 151] (also see Frasca et al., this volume). For example, Ikaros is expressed in all hematopoietic lineages and controls the emergence of lymphoid progenitors [68, 111]; the proto-oncogene PU.1 is critical for both myeloid and lymphoid differentiation [149]; and the transcriptional repressor Bcl11A is essential for normal B- and T-cell development [72]. The E protein family members E2A and EBF coordinately activate the expression of B-cell specific genes, especially those governing pre-BCR production and function [64, 66, 119, 150] and also regulate Pax-5 [140], a key mediator that activates B lineage-specific genes and represses genes associated with other lineages [16, 17]. IL-7/IL-7R is a key cytokine axis for early B lineage development, promoting both survival and differentiation [2]. Complex interplay between cell intrinsic and extrinsic signals characterizes the regulation of these transcriptional systems [26, 90, 150].

Although these transcriptional and signaling events occur prior to antigen receptor gene rearrangement, they can nonetheless influence repertoire composition in several ways. First, they dictate the rate of lineage commitment, thus impacting B-cell production rates and shifting downstream homeostatic demands. Second, they influence the rate and specificity of key intracellular events, thus coloring the likelihood and nature of heavy and light chain gene rearrangements.

Subsequent to lineage commitment, B-cell differentiative stages are characterized according to surface markers and Ig gene rearrangement status [46–48, 94, 121, 122, 138]. In the pro-B stage, cells rearrange their Ig heavy chain genes [40, 180]. This is followed by surface Ig heavy chain expression with surrogate light chain and BCR signaling molecules, delineating onset of the pre-B-cell stage. Surface pre-BCR expression and signaling are required for transit from the pro- to pre-B-cell stage [61, 92, 93, 123, 133, 134, 164, 165], and result in a proliferative burst characteristic of the large pre-B-cell compartment. Light chain gene rearrangement during the late pre-B-cell stage leads to the expression of a complete BCR, defining the immature (IMM) BM B-cell. Once in the IMM subset, cells either die or exit the BM to complete maturation [6, 7].

2.2 Peripheral Maturation and Primary B-Cell Subsets

Recent marrow émigrés have been dubbed transitional (TR) B-cells [73], and can be further divided into subsets termed T1, T2, and T3 [1]. While historically viewed as a linear progression from the IMM marrow stage through the successive TR compartments, it now appears that branched, asynchronous models for transit into and through these subsets are more likely [5, 91, 145, 153]. Cells that successfully complete TR differentiation enter the mature peripheral B-cell pools.

Mature follicular (FO) B-cells, also termed B2 or “conventional” B-cells, encompass the majority (>80%) of peripheral B-lymphocytes, and are the progeni-

tors of both primary antibody forming cells and memory B-cells. Additional subsets of mature B-cells include marginal zone (MZ) B-cells, which are phenotypically, functionally, and anatomically distinct from FO B-cells and play a major role in responses to T-cell independent (TI) antigens. In peritoneal and pleural cavities the B1 subset predominates (reviewed in [52]). B1 B-cells appear first in ontogeny and are maintained by self-renewal [48, 49, 53]. The MZ and B1 subsets share several functional attributes, particularly participation in TI immune responses [63, 74, 75, 83]. The derivation of B1 B-cells, though distinct from the other B-cell subsets, is not yet entirely clear [1, 14, 52, 54, 74, 104, 176]. However, in the context of age-associated repertoire shifts, it is noteworthy that while production from BM B2 progenitors predominates in young adults, this wanes in aged mice. Therefore, if B1 progenitors are stable and distinct from B2 progenitors, the B1 lineage may wax with advancing age; thus altering the combined B-cell repertoire [104, 105].

2.3 Selection and Homeostasis Among Emerging and Primary B-Cells

Although they occur before complete BCR expression and perforce cannot be specificity-driven, heavy and light chain gene rearrangement processes, as well as heavy chain selection at the pre-BCR stage, will influence the incipient B-cell repertoire. For example, only heavy chains with structural characteristics affording surrogate light chain pairing are selected for further differentiation [81, 95]. In addition, heavy and light chain gene rearrangement processes rely on multiple factors, including the expression of appropriate enzyme and targeting complexes, accessibility and marking of heavy and light chain loci, and the activity of DNA damage resistance and repair systems (for reviews see [32, 59, 62, 107, 108, 139, 142–144]).

The interaction of homeostasis and selection powerfully impacts all B-cell subsets downstream of BCR expression, directly determining repertoire composition. A critical notion emerging from appreciation of this interplay is that events acting upstream of mature B-lymphocyte pools can impact downstream populations. Since advancing age is accompanied by substantial shifts in both B-cell generation and the success rate of IMM and TR differentiation, distinguishing primary lesions from homeostatic compensation is critical to a mechanistic understanding of age-related changes [19, 99, 131].

Stringent specificity-based selection occurs at the IMM stage, where high avidity interactions yield secondary Ig gene rearrangements or death [21, 22, 41, 109, 110, 115, 116, 128–130, 162]. These central tolerance mechanisms result in the loss of ~90% of all IMM cells formed [7, 120]. While it is possible that the IMM pool is governed by homeostatic mechanisms to preserve its size, this remains speculative and does not seem tied to BCR-mediated negative selection. Instead, BCR signal strength is the major, if not sole, determinant of survival at the IMM stage.

Specificity-based selection continues to act on newly formed cells that exit the marrow to join TR pools. Whereas high avidity BCR interactions lead to cell death, a lack of minimal BCR signaling precludes maturation and entrance to mature

peripheral pools [20, 23, 33, 51, 82, 169]. While BM negative selection depends on BCR signal strength (and is therefore cell-intrinsic), the likelihood that a given cell completes TR differentiation to join a mature B-cell subset is based on both BCR signal strength and the availability of B-lymphocyte stimulator (BLyS, also termed BAFF). BLyS is the limiting resource for which TR, FO, and MZ B-cells compete (reviewed in [18] and [100]). Through this competitive mechanism, steady state numbers of mature B-cells are governed by ambient BLyS levels that vary the proportion of TR cells completing maturation, as well as the lifespan of FO and MZ B-cells [50, 57]. This connection between BCR specificity and fitness for interclonal competition indicates that BLyS availability, within the context of the emerging clonotypic cohort, will determine thresholds for TR selection. This relationship has recently been confirmed in several transgenic systems [56, 70, 161].

The relationship between BLyS availability, antigen receptor specificity, and TR selective stringency makes several implications relevant to age-associated changes in the primary repertoire. First it implies that BCR- and BLyS-mediated signals must be integrated, possibly via cross-talk between intracellular signaling systems [154]. Although the molecular details remain the subject of intense research, age associated perturbations of any of these systems may influence primary repertoire diversity. Moreover, decreased B-cell generation rates in BM—a feature of the aging immune system—might permit a broader array of clonotypes, including autoreactive cells, to enter peripheral pools as competition wanes [100].

3 Antigen-Experienced Pools

3.1 Establishing and Maintaining Antigen-Experienced Subsets

Antigen-experienced subsets contain the descendants of primary B-cells recruited into immune responses, and thus include activated cells themselves, as well as the resulting effector and memory pools. Humoral immune responses are generally characterized as T-dependent (TD) or T-independent (TI), depending on their requirement for cognate T help. In general, protein antigens engender TD responses, reflecting the requisite for MHC class II restricted presentation that affords delivery of costimulation. These responses primarily involve FO B-cells, and typically lead to long-term humoral immunity. A key characteristic of TD responses is the formation of germinal centers (GCs), where proliferating B-cells undergo class switch recombination, as well as the somatic hypermutation (SHM) and affinity-based selection processes that culminate in cells producing high-affinity antibody [86]. In contrast, TI responses do not require cognate help, although T-cell derived cytokines may promote limited isotype switching [15]. TI responses elicit little if any memory, lack substantial hypermutation or affinity maturation, and consist predominantly of IgM. Two classes of TI antigens exist: TI-1 responses are induced via pattern recognition receptors [148]; whereas TI-2 responses are generated by antigens with densely repeating epitopes. Both TI-1 and TI-2 responses preferentially arise from

B1 and MZ B-cells. Whether this reflects intrinsic bias in the pre-immune repertoires of these cells or more extensive expression of pattern recognition receptors [58, 84]—both of which are empirically observed—remains debated.

Antigen activation yields a series of short-lived cells, which are detectable for only days or weeks following antigen challenge; as well as several subsets of long-lived cells, which persist for months or years [80, 141, 163]. During TD responses, antigen activated B-cells become either GC B-cells, or short-lived plasma cells (PC), in a differentiation decision dictated by BCR affinity [124]. Short-lived PC arise in the first few days of an immune response, congregating at the T/B-cell interface and extrafollicular regions of secondary lymphoid organs [86]. The critical relationship between BCR repertoire and recruitment into long-lived pools has been revealed using transgenic systems [24, 25]. For example, B-cells with low initial affinity for antigen can participate in GC reactions when higher affinity competition is eliminated, suggesting that initial repertoire can shape the pool of antigen-reactive B-cells that ultimately succeed and contribute to immune responses. Long-lived antigen-experienced populations include a group of long-lived PC, as well as a separate group termed memory B-cells [79, 87]. The delineation of these groups based on surface markers is debated; however, a clear functional difference is that long-lived PC secrete antibody, while memory cells do not [9, 28, 78, 85, 87–89]. The lineal relationships between various antigen-experienced subsets are unclear. For example, whether long-lived populations are generated from cells within the generally short-lived populations, or instead differentiate from distinct progenitors through a separate selective mechanism, is debated.

3.2 Homeostasis in Antigen Experienced Subsets

The concept of a biological niche for naïve B-cells is well established, with the BLyS/BR3 ligand/receptor axis playing a central role. In contrast, knowledge of factors governing the size and composition of antigen-experienced B-cell subsets is more limited. As with naïve pools, interplay between homeostasis and selection seems likely in the establishment and maintenance of antigen-experienced subsets. Multiple steps in the generation of effector and memory B-cells rely on selective decisions. These include the relationship between BCR affinity and recruitment into the extrafollicular PC pool versus the GC [124]; affinity maturation per se; as well as commitment to long-lived PC versus memory B-cells [8, 31].

Homeostatic controls, while evident in antigen-experienced pools, also remain poorly understood. While neither short-term effectors or long lived antigen experienced populations compete with primary B-cells for survival, the trophic factors and relationships are only now being explored. Recent evidence suggests that additional BLyS family receptors or ligands, such as TACI, BCMA and APRIL likely play a role. In support of this idea, TACI is associated with activated B-cells and regulates some TI immune responses [163, 168], whereas BCMA is required for survival of long-lived PC in BM [118]. Further, ongoing immune responses appear to create

temporary homeostatic niches for short-lived populations, while leading to little change in long-term protective memory pools [132]. Finally, long-lived BM PC survival is competitive [78–80], possibly involving cell extrinsic stromal factors, as well as Fc-gammaRIIb expression [177].

4 Age-Associated Changes in Progenitor and Primary Pools

An extensive literature suggests the primary repertoire shifts with age. For example, the phosphorylcholine-specific repertoire shifts from one predominated by the T15 clonotype to a more diverse pool [113, 114, 136, 185]. On the other hand, overall diversity in the primary pool is not altered extensively, as assessed by fine specificity analyses [113, 183]. Nonetheless, clonal expansions in both the B and T-cell pools suggest that some specificities can be inordinately expanded. Understanding the basis for these changes requires considering all events likely to impact repertoire generation, selection, and maintenance. These include changes in the size and behavior of generative pools, as well as changes in the primary pools themselves.

4.1 B-Cell Generative Rates and Subsets Change with Age

Age-associated changes in B lineage development include reductions in precursor frequencies, lowered expression of critical regulatory genes, diminished pro- and pre B-cell numbers, and damped responsiveness to differentiation cues [35, 39, 60, 69, 97, 136, 146, 156, 157, 166]. Together, these observations indicate overall diminution of B-cell generation and throughput.

The impact of aging is first manifested in HSCs and CLPs. Somewhat paradoxically, while HSC numbers are maintained and possibly expanded in aged mice [179], the MPP/ELP and CLP pools are reduced [3, 97, 98]. Although the basis for this remains unclear, correlations with age-associated reductions in stromal IL-7 production [156]; as well as reduced expression of E2A and EBF and genes they control, suggest these may contribute [35, 39, 147, 166, 167]. As might be expected from these changes in upstream pools, pro-B-cell numbers are reduced, with an even greater proportional reduction in pre-B-cell numbers [4, 131, 135]. This decline in part reflects reduced IL-7-mediated proliferation at the pro- to pre-B transition [103, 155]. Hormonal changes may be another important extrinsic factor, since pregnancies delay the age-associated reduction in BM B-cell production [12].

The dynamics of developing B-cells also change with age. In vivo BrdU labeling studies [60, 67, 69] showed reductions in successful pro- to pre B-cell transit, yielding a fourfold drop in pre-B-cell numbers, and a corresponding decrease in the IMM B-cell generation rate. However, the throughput of pre-B-cells increased, so a twofold greater proportion of pre-B-cells enter the IMM pool. In addition, residency

within the IMM pool is longer. Together, these apparent compensatory features result in an IMM pool that is only about twofold smaller than in young adults.

4.2 Dynamics and Proportions of Peripheral Subsets Change with Age

Reflecting the upstream reductions in IMM B-cell numbers, TR pools are reduced in throughput and size; however, because residency time is extended, TR cell numbers are not significantly reduced. Similarly, the FO pool's turnover rate is reduced in aged mice [60, 67, 131], so FO pool size is maintained in the face of reduced marrow production. Despite this fairly stable overall size, B-cell clonal expansions are more prevalent in aged mice [13, 171, 172]. In contrast, the MZ and B1 pools are unaffected or even enlarged in aged mice, but this may vary by strain [4, 131, 171].

Whether the homeostatic mechanisms controlling primary B-cell numbers change with age has not been extensively interrogated. However, several recent observations suggest this is likely. For example, in young adults, emerging cells expressing high levels of the BLyS receptor, TACI, are selected during TR differentiation. This process is dampened in aged mice, allowing cells with lower TACI levels to join the mature FO pool [131]. In addition, FO B-cells from aged mice more effectively capture BLyS-mediated survival signals in vitro, although the underlying mechanism is unclear. These observations suggest a model whereby selection at the marrow-periphery interface is relaxed in aged mice; yet competition among mature B-cells may be more severe, reflecting lifelong selection for optimally fit clonotypes [131].

4.3 Developing and Primary Repertoires Change with Age

Alterations in the BM pre-selection repertoire might be expected, given the numerous age-associated changes in cytokine and transcription factors, many of which are involved in Ig gene rearrangement [39, 69, 146, 147, 156]. There is a correlation between the age-associated reduction of pre-B-cell numbers and reduced RAG gene expression, V(D)J recombinase activity, and V to (D)J rearrangement [69, 159, 160]. Evidence for age-associated, intrinsic shifts in V gene segment use are suggested by studies showing an increased frequency of phosphorylcholine-responsive cells arising from sIg^- BM cells in aged BALB/c mice [185]. These increases included clonotypes bearing VhS107 (T15) as well as other Vh segments.

The interplay of intrinsic and microenvironmental changes in aged BM could affect the pre-selection repertoire in several ways. Shifts in heavy chain allele choice at the pre-BCR stage or in light chain choice at the pre-B stage could alter repertoire

composition. Decreased pre-B-cell production may mean that fewer B-cells of different clonotypes are generated. However, this effect may be at least partially offset if extended residency time in the IMM stage affords greater opportunity for receptor editing or Vh gene replacement. Finally, the existence of multiple B differentiation lineages whose Vh gene preferences differ and whose dominance varies with age might underlie some of these observations.

There is ample evidence for age-associated shifts in the primary repertoire, but whether these act to generally expand or contract diversity is uncertain. The phosphorylcholine-specific response in young BALB/c and B6 mice is dominated by VhS107/Vk22 gene segments, whereas aged mice use a broader range of Vh and Vk segments [112, 113]. Moreover, phosphorylcholine-binding monoclonal antibodies generated from aged mice show greater polyreactivity. In contrast, while the frequency of NP-responsive cells is about twofold lower in aged mice, there is no accompanying change in repertoire diversity or clonotype distrubution [184]; and repertoire diversity to influenza hemagglutinin is similar in aged and young mice [183]. Finally, the autoreconstituting repertoire that emerges after irraditaion- or drug-induced lymphopenia is truncated in aged mice, when assessed by CDR3 length heterogeneity [71].

5 Immune Responses and Antigen-Experienced Pools Change With Age

Some age-related changes in immune responses may be related to shifts in the preselection or primary repertoires, while others may be the result of alterations that are observed as or after responding cells have encountered antigen. Immune responses in aged mice sometimes—but not always—involve reduced antibody production and/or antibody of lower affinity in comparison to young mice; however, overall diversity of the responding repertoire is retained or enhanced. Short-lived PC responses and pools are normal to increased in aged individuals, whereas long-lived PC and memory cell numbers are reduced. All of this suggests that the antigen-experienced repertoire is different in quality and possibly quantity in aged mice.

Extensive age-associated changes have been reported in TD immune responses. These include impaired GC formation and kinetics, defective cellular interactions, and deficiencies in SHM and affinity maturation (reviewed in [187]). The antibody response to NP-CGG in aged B6 mice is impaired in terms of primary response kinetics and the amount of antibody produced; moreover, the average affinity of NP-binding antibodies is sixfold lower than in young mice [96]. Although GCs form in aged mice, their number and size are significantly reduced, their kinetics are delayed, and there is no detectable SHM. In apparent contrast, some experimental systems suggest that SHM yields increased diversity of serum Igs in aged mice [175]. These different results are not necessarily contradictory, as SHM may occur even when B-cells are activated outside of GCs [173, 174]. Microenvironment may play an important role: Peyer's patch GC B-cells from aged B6D2F1 mice were

similar in frequency and activation phenotype to those observed in young mice, yet showed higher somatic mutation frequencies [137].

Cellular interactions are also altered with age [43, 152, 178]. T-cell intrinsic changes may account for some of this; for example, a decrease in IL-2 production with age leads to reduced CD40L expression as well as a general CD4+T-cell population shift away from a naive phenotype and towards either a memory or a regulatory phenotype [55]. The proportion of antigen-responsive B-cells to DNP-specific stimulation is decreased in aged mice, and T-cells from aged mice can down-regulate B-cell responsiveness [181, 182]. In an Igh^b *scid* chimera system with donor lymphocytes from young or aged mice, where the primary response to NP is highly restricted to use of Vh186.2/lambda-1 gene segments, aged donor helper T-cells—but not aged B-cells—are less effective at inducing GC formation, and shift Vh gene segment use away from Vh186.2 to include higher proportions of others, particularly C1H4 [178]. In addition, SHM in GC B-cells was reduced in frequency with aged donor T- or B cells. Thus, both germline repertoire use and SHM are likely altered in aged mice; and immunosenescence likley results from changes in both B- and T cell compartments.

Class-switch recombination also appears impaired in aged mice [36–38]. B-cells from old BALB/c mice stimulated in vitro with optimal levels of CD40L and IL-4 display a reduced ability to isotype switch [38]. Defects in isotype switching as well as SHM are associated with an age-related downregulation of E47, which leads to reduced expression of the activation-induced cytidine deaminase (AID) [37].

Mirroring the spectrum of observations in primary repertoire analyses, whether age impacts the magnitude or diversity of antibody responses depends on the model antigen employed, as well as the strain of mice studied. For example, the magnitude of the antibody response to *S. pneumoniae* vaccine and TNP-BA differ in B6 and BALB/c mice, indicating a role for genetic factors in the immune response [113]. However, the clonotypic diversity of the response to both antigens and to phosphorylcholine is greater in aged mice of both strains [112, 113]. In contrast, both primary and secondary responses to DNP-BGG are reduced in aged mice [43, 170]. A study of the IgM component of the primary response to TNP-KLH shows that the peak IgM response is delayed in aged mice but the spectrum and affinity of antibodies are similar to those seen in young animals [186].

Several studies have shown decreased affinity or avidity of antibodies produced by aged mice in TD responses, in some cases along with evidence for a role for altered T-cell responses [27, 43, 114, 170, 186]. When mixtures of phosphorylcholine-specific antibodies from young or aged donor mice are injected into recipients that subsequently receive a lethal dose of *S. pneumoniae*, only antibodies from young donors allowed survival [114]. Moreover, the average affinity of antibodies from aged donors is lower than that of young donors for free PC hapten [114]. Thus the efficacy of antibodies produced by aged mice may be quite different from those produced by young mice. In accord with this overall picture, aged mice challenged with NP-CGG show a higher antigen-forming cell (AFC) response than young mice, but smaller and fewer GCs [45]. Most of the AFC in old mice were low-affinity IgM producers, and the number of high-affinity AFC

was half that of young controls. There were significantly fewer AFC in BM of aged mice following immunization, and reconstitution experiments demonstrated that aged BM was defective in supporting AFC. Thus, the spleen may prove the primary source of the antibody response in aged mice, in contrast to BM in young mice. This shift in AFC location could reflect several potential age related defects in the B-cell response including potential BM homing problems, an altered antigen specific precursor frequency, reduced capacity for AFCs in the BM environment, or a combination of these and other factors. Due to the unclear relationship between naïve B-cells and long-lived PC generation, it is difficult to determine whether impaired humoral immunity in the aged is due to a failure to generate cells capable of seeding the BM and becoming PC memory, or if the defect is downstream. It has been proposed that long-lived PC occupy a highly specialized, tightly regulated niche, and it is possible that this niche is unable to support the entrance of newly formed long-lived PC in old mice, due to intensive competition for survival factors [132].

Only a few studies have addressed TI responses in aged mice. Zharhary [186] made a direct comparison of the IgM response following immunization with TD versus TI forms of the TNP hapten. While the peak IgM response was delayed in aged mice for the TD antigen, there was no delay for the TI antigen. Similarly, Weksler [170] reports that TI responses are generally less impaired than TD responses in aged mice.

It is tempting to speculate that because TI responses are largely B-cell-intrinsic, they will be less severely impacted by age-related changes in T-cell function. Moreover, TI responses may be further preserved by the age-associated persistence of MZ and B1 cells [131], which are largely responsible for antibody production to TI antigens. Thus, the comparative resilience of TI responses may increasingly impact repertoire composition with advancing age.

6 Summary and Perspective

Assessing the nature and basis for repertoire changes is a first-order consideration in our understanding of immunosenescence. Multiple processes appear to act in concert to alter repertoire with age. These include reduced B-cell generation, shifts in V gene choice, and altered subset dynamics and selection overlaid with compensatory homeostatic mechanisms. Murine model systems are attractive routes to interrogate the underlying mechanisms, not only because of their substantial similarities to age associated shifts in human immune responsiveness, but also because they provide an opportunity to approach basic questions experimentally. Some important questions include why and how BM B-cell generation decreases with age, and how this impacts repertoire; whether the stringency of central or TR tolerance change with age; and how B-cell repertoire shifts and impaired immune responses in aged individuals are linked.

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B-Cells and Antibodies in Old Humans

Kate L. Gibson and Deborah K. Dunn-Walters

Contents

1 Role of B-Cells in Age-Associated Susceptibility to Infection

It has been well established that the efficiency of the immune system declines with increasing age. Immunosenescence causes increased susceptibility to infectious diseases, and infection is, in fact, the third leading cause of mortality in people aged 65 and over [1]. As is clearly apparent from the other chapters of this book, there are many components of the immune system that can change with age, and are crucial to maintaining an effective immune system. The humoral immune system interacts with the other components, both as part of its own development and via its effector mechanisms. The most important function of B-cells is to produce antibodies, the indispensable soluble effectors of many functions. There are a number of different stages of development for B-cells and their antibodies (Fig. 1).

In the primary B-cell response antibodies that recognize pathogen, although not necessarily with high affinity, are rapidly produced. They may include the so-called "polyspecific" antibodies, which have the ability to recognize multiple antigens [2]. The first antibodies are of the IgM isotype and are crucial for opsonizing pathogens, inducing phagocytosis and activating the complement cascade. These

D.K. Dunn-Walters (✉) · K. L. Gibson
Department of Immunobiology
2nd Floor, Borough Wing Guy's
King's and St. Thomas School of Medicine
King's College London
Guy's Hospital, Great Maze Pond
London SE1 9RT
E-Mail: Deborah.Dunn-Walters@kcl.ac.uk

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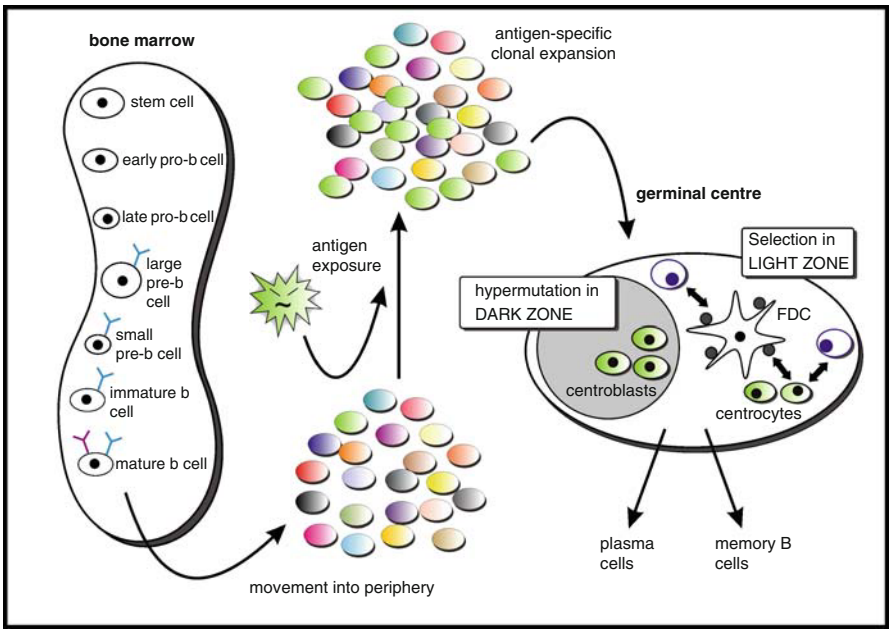

Fig. 1 B-cell development. The humoral immune response is mediated by antibodies produced from plasma cells. These plasma cells are the end point in B-cell development, which is characterized by (a) generation of a huge diversity of different B-cells, each carrying a different antibody gene in the bone marrow and (b) selection processes using the affinity of the membrane-bound form of the antibody (the B-cell receptor) for it's antigen as the selection criteria. Diversity is generated by a process of gene rearrangement early on in the development of the cell, in the bone marrow prior to antigen encounter. The selection processes are twofold. Firstly B-cells are selected for survival, or not, on the basis of their antibody recognition—to eliminate inappropriate self-reactivity and encourage reactivity with foreign pathogens. Secondly there is a mutation step in development, and the resultant B-cells carrying improved antibodies are selected—this occurs in the germinal centre of secondary tissues, after encounter with antigen, and serves to increase the affinity of the antibody for the relevant antigen. Both generation of diversity and selection of antibody are complex processes that are crucial for an effective humoral immune system. A clear understanding of these processes, and how they are affected with age, is needed in order to comprehend the etiology of age-related inflammatory and infectious disease

antibody functions, and the rapidity of this primary response, have been shown to play a vital role in protection from extracellular bacterial pathogens [3]. Antibodies afford protection against viral infection by neutralizing the virus particles; binding and blocking key molecules involved in cellular infection. Similarly they can also neutralize toxins. Later maturation of the B-cells in the immune response is slower but results in the generation of more highly specific antibodies, which may be of a different isotype, following a process known as affinity maturation. In addition to the neutralizing and opsonizing functions of antibody, B-cells are also important as modulators of inflammation [4, 5], regulators of the immune response [6] and as antigen presenting cells and activators of T-cells [7–10].

The elderly are susceptible to infections by a wide variety of pathogens, all of which involve B-cells and antibodies in the normal course of the immune response (Table 1). The lungs are, in common with other mucosal surfaces of the gastrointestinal and genito-urinary tracts, particularly vulnerable to infection by virtue of their exposure to the environment. As is illustrated in Table 1, pulmonary infections are common in older people. The elderly are usually the first to be affected by annual epidemics of respiratory infections, and frequently suffer the worst clinically. Mortality figures attributable to influenza and pneumonia are confused by the fact that influenza is very often followed by a secondary infection—most notably by *Streptococcus pneumoniae*. Some would argue that this confounding factor results in a two to threefold underestimate of influenza mortality [23]. It is also argued that mortality due to influenza is negligible and it is the secondary bacterial infection that causes almost all deaths [24, 25]. Whichever way round, it is generally agreed that older people are the worst affected by these diseases. It has been reported that 90% of all pneumonia and influenza deaths and 88% of respiratory syncytial virus-associated deaths occur in those aged over 65 years [26]. In the oldest old (85 years and over) there was a 32-fold increased chance of mortality from influenza or influenza-associated pneumonia compared with those aged 65–69 years [26]. According to the Department of Health, in the UK there are more than 18,000 hospitalizations resulting from pneumococcal pneumonia each year in those aged 65 years and over [27]. There is also an increased incidence of pneumococcal septicemia in old people associated with *S. pneumoniae* infection [28].

Table 1 Pathogens found frequently in elderly subjects with respiratory or urinary tract infections. (adapted from [1])

Organ system	Pathogen found frequently	B-cell role in immune response to pathogen
Respiratory tract (upper and lower)	Bacteria	
	Streptococcus pneumoniae	B-cells are crucial to the TI-II response [11]
	Hemophilus influenza	Mucosal IgA has a protective role independent of serum antibody levels [12]
	Legionella pneumophila	B-cells are required for opsonization [13]
	Chlamydia pneumoniae	Neutralization by antibody [14]
	Viruses	
	Rhinoviruses	Antibody-mediated neutralization [15,16]
	Coronaviruses	
	Influenza	
	Respiratory syncytial	
Urinary tract	Bacteria	
	Escherichia coli	IgA secretion and antigen-specific Ig inhibits attachment of bacteria [17,18]
	Proteus	An increase in IgM and IgA aids protection [19,20]
	Klebsiella	
	Pseudomonasaeruginosa	Opsonization [21]
	Enterococci	Antibody alone not hugely effective, but effective in the presence of complement [22]

It is known that specific antibodies, generated during a T-dependent B-cell response, are crucial for protection against influenza. Ineffective influenza-specific antibody, as assessed by the Haemagglutination inhibition (HI) test, is associated with lowered protection from the disease [29]. Studies have shown that 25% or more of the elderly fail to develop HI titres of a protective level following vaccination [30, 31]. In vivo studies in mice have shown that higher levels of B-cells and IgG2a antibody confer increased levels of protection [32]. It has been said that an age-related decrease in influenza protection can be solely accounted for by the reduced T-cell help available in the diminished elderly T-cell repertoire. However, this does not take into account the fact that the CD4+ T-cells themselves may rely on fully functioning B-cells for their activation [7, 10].

In other areas of humoral immunity the B-cells are even less reliant on T-cells for help. Pneumonia is a bacterial infection, caused by a number of different organisms (e.g. *Streptococcus pneumoniae* [33], *Staphylococcus aureus* [34], *Streptococcus pyogenes* [35]) although *S. pneumoniae* is the major cause [33]. Immunity against *S. pneumoniae* is particularly reliant on a healthy B-cell population. This is because the antigenic portion of *S. pneumoniae* is a capsular polysaccharide and a T-independent type II (TI-II) antigen. Unlike a T-dependent B-cell response, where the maturation of the B-cell antibody relies on T-cell help and therefore any failure to respond could be attributed to a failure of T-cells, the TI-II response is independent of direct T-cell help. Therefore a failure to protect against *S. pneumoniae* is more likely to be a failure ascribable to deficits in the B-cells themselves.

In children a reduced pneumococcal response can be explained by a lack of marginal zone B-cells in the spleen, where the main TI-II responding B-cells are thought to reside. However, older people appear to have a fully functioning splenic marginal zone [36] so the lack of effective pneumococcal protection in the elderly still remains a mystery. One good candidate for further study is the IgM response. It has been shown, in mice, that the classical complement pathway, partially mediated by binding of natural IgM to bacteria, is vital for innate immunity to S. pneumoniae [3]. Human studies have also shown that antibody of the IgM isotype is vital in providing efficient protection against S. pneumoniae [37], although this has been mainly attributed to "IgM memory," with mutated IgM genes. The exact roles and relationships between natural antibody, IgM memory and class switched memory in the pneumococcal response remain to be determined.

The immune response of the elderly to RSV is less well studied than that against other pulmonary infections. Recent data shows that the senescence accelerated mouse has a severely compromised cellular immune system and produces less virus-specific local IgA in response to RSV infection [38].

Although pulmonary infections of the elderly are the most notable, by virtue of the fact that they cause the most mortality, there are also significant increases in morbidity and mortality from other infections. Bacterial infections of the skin, urinary tract, soft tissue, and gastrointestinal tract are all increased with age [1]. The exact role of the humoral response in this declined protection has yet to be elucidated.

2 Vaccination in the Elderly

Vaccines are an extremely important tool in preventing deaths from infection, and since they are routinely administered as part of a normal health care routine they are the main source of data on immune responses in man. It has been consistently shown that the effectiveness of vaccines is severely diminished in older people. The most commonly studied vaccine is that against influenza. The cellular response, i.e. T-cells and release of cytokines, macrophages and natural killer cells, is decreased with age [39]. In terms of the humoral response the antibody titre, in the form of IgG, is significantly lower [39–41]. While vaccination of the elderly against influenza is widely accepted as a valid health strategy to reduce disease incidence, and studies support this, [42–44] other studies suggest that influenza vaccination does not significantly decrease influenza-related mortality in older people [45, 46]. The age-related reduction in specific antibody production also occurs in response to other vaccines, such as against hepatitis B [47], tetanus and tick-borne encephalitis (TBE) [48]. Data on some of the less common vaccines is more scarce, but gradually becoming available with the advent of an older population which travels more widely. Some travel vaccines, such as hepatitis A, also show a reduced specific antibody response [49], while others such as yellow fever seem to show an undiminished antibody response but have an increased risk of adverse events in the elderly [50].

A possible explanation for a decrease in specific antibody is that the process of affinity maturation is defective. During one study on influenza vaccine it was discovered that an age-related decrease in specific antibody was accompanied by an increase in antibodies against double stranded DNA—indicative of self reactive/polyclonal B-cells [51]. Polyclonal B-cells are often associated with naive B-cells that have not been through the affinity maturation process and are reacting in either a low-affinity manner to specific antigen, or in a non-specific manner by virtue of their innate pattern recognition responses. It was this finding that led to the idea that perhaps humoral immunity in the older person was better represented by the T-independent response. However, as mentioned above, there is a large T-independent component to immune protection against *S. pneumoniae* and general protection is decreased with age. Cross-reactive antibodies certainly appear to be increased in older people treated with the polysaccharide pneumococcal vaccine [52], although the failure of the vaccine to adequately protect against pneumonia [53–57] implies that they are not adequate compensation for the reduction in specific antibody that is also seen [52].

3 Autoantibodies and Age

There is a well-documented shift towards self-reactive antibody production with age. One of the most common autoantibody types, frequently associated with disease, is antinuclear antibodies (ANAs). These have consistently been found to be

increased in the old (over 65) in the absence of disease; a prospective study showed persistence of these raised levels throughout older life [58]. The significance of this increase has not yet been determined, and attempts to relate these antibodies with general levels of disease and frailty have shown no associations. The Swedish longitudinal NONA immune study [59] showed significantly higher ANA levels in the oldest old (86–95 years) but found there to be no association nor any correlation to other immune risk factors (e.g. CD4/CD8 T-cell ratio, CMV seropositivity). These findings are echoed by a Finnish study, where ANA positivity at the age of 90 did not show any correlation with survival, or with the levels of serum markers of inflammation [60]. It has even been suggested that an increase in ANA antibodies may have beneficial effects by virtue of a possible anti-tumor activity [61].

ANAs are not the only auto-antibodies to increase with age. The study by Xavier et al. [58] also noted an increase in the frequency of anti-ssDNA antibodies, as have other studies [62, 63]. Increases in antibodies against many other auto-antigens have been reported, for example against cardiolipin, dsDNA and rheumatoid factor, [62–65] although, again, there were no associations found with mortality [62]. The Danish study by Andersen-Ranberg et al. [65] did find a correlation between autoantibodies and comorbidity and disability, although this was only for the organ-specific antibodies, indicating that these were more likely a result of age-associated disease.

Although the aetiology of Rheumatoid arthritis (RA) is not yet fully elucidated, it is an age-related inflammatory autoimmune disorder. Coincidentally, as reported above, there is also an increased incidence of rheumatoid factor (RF) with age—regardless of whether the subject has RA or not [62–65]. There has been a decline in incidence of the disease that has been observed over the last 40 years [66] which has been attributed to environmental factors. One possible contributor to this is the gradual decrease in the number of smokers. Recent evidence has shown that the presence of another auto-antibody, anti-cyclic citrullinated peptide (anti-CCP) is associated with smoking and a higher risk of RA [67]. The successful use of therapies such as Rituximab, which utilize an anti-CD20 monoclonal antibody to ablate peripheral B-cells, is ample evidence that B-cells play an important part in the disease process of RA [68]. In addition to the obvious mechanism of depleting auto-antibody producing cells, there is increasing evidence for a role of B-cells in RA as antigen-presenting cells, activating T-cells, and producing and responding to cytokines [69]. A further complication in understanding the role of B-cells is the fact that B-cells have recently been shown to be capable of immunosuppression—including in animals models of arthritis [70, 71].

4 Immunodysregulation of B-cells in Aging

The above observations are all evidence that the humoral immune system is dysregulated in older people. At first glance it would appear that there is no easily identifiable quantitative defect in the humoral immune system with age. However, although the range of B-cell numbers, as a percentage of peripheral blood lymphocytes, varies greatly between individuals, it has been reported that there is a slight decline in the

number of CD19+ B-cells in old age [72–75]. It has also been reported that having a higher number of CD19+ B-cells is associated with better survival [76, 77]. When CD20 is used as a marker for B-cells no age-related change could be found [78]. The number of antibody molecules circulating in the periphery of older adults remains relatively stable [79, 80]. Similarly, studies have been conducted on the ratio of different Ig isotypes in the elderly and most show no significant change during later life [75, 81, 82]; although it has been reported that an increase of the mucosal IgA antibody in the serum can be a predictor of mortality [83]. In general the picture is one of a qualitative change in the antibody repertoire rather than a quantitative one [84].

4.1 Generation of High Affinity Antibodies

Since the lack of high affinity antibodies is a key feature of the older immune system, and our expertise is in the study of Ig genes, we initially investigated the affinity maturation process. Affinity maturation occurs in the germinal centre (GC) and involves the expansion of antigen-specific B-cells, mutation of their Ig genes (resulting in altered antibody function), followed by selection of the B-cells producing the best antibody [85–87]. Contained within the dynamic microenvironment of the GC are B-cells, T-cells, and follicular dendritic cells (FDCs) all in close proximity to allow the exchange of costimulatory molecules and cytokine signaling.

Following antigenic stimulation, selected B-cells migrate and converge on the GC FDCs, making contact with their long processes [88] and differentiating into centroblasts. The FDCs are the stromal cells of the GC and play a key role in regulating the humoral immune respone [89]. Unlike antigen presenting cells (APCs), FDCs present intact antigen–antibody complexes on their cell surface [88], in the form of immune complexes which are highly immunogenic, and assist GC B-cell proliferation [90–92]. Proliferating GC B-cells are known as centroblasts. During centroblast proliferation, in the dark zone of the GC, hypermutation of the immunoglobulin (Ig) genes encoding antibody occurs. The B-cells move into the light zone, as centrocytes, and will die through apoptosis unless they receive rescue signals conditional on efficient recognition of the antigen by the newly formed B-cell receptor. Rescue signals are provided by FDCs and T-cells [93]. The helper T-cells in the GC are a particular subset of CD4+ T-cells, expressing CD57. These cells have unique characteristics that have yet to be fully elucidated [94]. Since FDC and T-cell help is limiting there is competition between B-cells and therefore selection of those B-cells with the highest affinity for antigen occurs. The resulting B-cells can switch the class of their antibody, from IgM to IgG/IgA/IgE, and this also requires T-cell help. B-cells with high affinity antibody differentiate into either memory B-cells, to provide for an efficient recall response, or plasma cells to secrete antibody. We have addressed the possible age-related changes in the GC reaction in three main areas: proliferation of B-cells, hypermutation of the Ig genes, and selection of high-affinity, antigen-specific, antibodies.

4.2 Proliferation

A defect in B-cell proliferation would have severe consequences for the GC reaction, since the loss of cells due to deleterious mutations acquired by hypermutation is extremely large and the pool of B-cells required to counter this is therefore also large. For some cell types proliferating cells can reach replicative senescence—where the telomeres at the ends of the chromosomes erode at each division and therefore there is a limit to the amount of proliferation one cell line can undergo set by the length of the telomere [95]. It has been shown that telomere length decreases with age in T-cells, and to a lesser extent in B-cells [96, 97]. However, we do not believe that the proliferative capacity of B-cells in the GC is impaired in this way as a result of old age. Telomerase, the enzyme that elongates telomeres, is upregulated in the GC, being high in centroblasts and higher still in centrocytes. This results in B-cells leaving the GC for the periphery with substantially longer telomeres than when they first entered, up to 4 kb longer as determined by Southern blotting [98]. Further to this, memory B-cells have telomeres on average 2 bp longer than naïve B-cells [97].

There has been much debate as to whether the overall size and number of GCs decrease with age. Several studies have pointed to this though they have all been conducted in rodent models [99–101]. Immunohistochemical studies measuring the size and overall number of B-cell follicles in human spleen, Peyer's patches [36] and lymph nodes [78] have not shown any age-related difference. However, there have been two studies of human tonsil, performed by flow cytometry rather than measuring individual GC sizes, which have both reported a decrease in GC B-cells with age [99, 102]. Tissue specific differences may account for these discrepancies and further work would be needed to clarify the issue.

4.3 Hypermutation of B-Cells

As outlined above, somatic hypermutation occurs following activation of the B-cells by antigen and entry into the GC reaction. The mutations introduced are generally point mutations, though some insertions and deletions may occur, and tend to be in areas containing hotspot motifs [103–105].

There is conflicting opinion regarding whether there is a quantitative change in hypermutation in the ageing individual. Reports have indicated no change [106–108], a decrease [109, 110] or increase [99, 111, 112] in mutation with increasing age. The fact that these studies do not agree is hardly surprising as they do not take into account patient health history i.e. prior immune responses. The tissue origin of samples can also make a significant difference to the number of mutations observed, for example we have shown consistently that B-cells of mucosal origin have a higher level of mutations than those from, say, spleen or blood [113].

We addressed these issues by attempting to quantitate the frequency of hypermutation in individual B-cell GC expansions. We microdissected histologically-defined

areas of GC from the spleen and Peyer's patch follicles of young and old humans so that only the mutations in that particular GC reaction were counted [114, 115]. Individual B-cell expansions were identified by their Ig gene characteristics; by identifying Ig gene sequences that have the same CDR3 region we can identify related B-cell clones (Fig. 2, *see* later for a more detailed explanation of Ig gene rearrangement). Furthermore, we can draw a lineage tree of individual B-cell clonal expansions (Fig. 3) by analyzing the order of accumulation of mutations in the hypermutation process [114, 115]. In this way we look at the number of mutations that occurred within that particular clonal expansion, and can compare lineage trees from subjects of different ages. We have shown that there was no difference in the frequency of mutation occurring in human GC reactions in the spleen and Peyer's patch with age.

4.4 Selection of High Affinity B-Cells and Class Switching

Lineage tree construction can furnish information on the affinity maturation dynamics by measurement of lineage tree shape parameters. The shape of the lineage tree can help indicate the degree of selection that has taken place. For instance, a 'pruned' tree (few branches) indicates high selection pressure whereas a 'bushy' tree (many branches), indicates less selection (Fig. 3). Since a failure of adequate selection

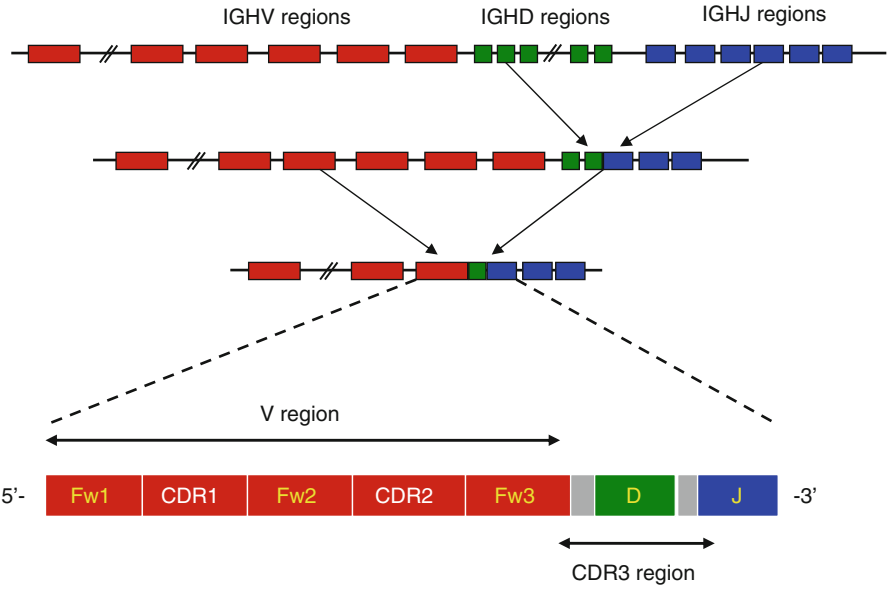

Fig. 2 Immunoglobulin heavy chain gene structure and the complementarity determining region (CDR) 3 region. The rearranged immunoglobulin gene contains 3 CDR regions (that form the antigen binding site) and 3 framework (Fw) regions (that provide structural integrity). During germline Ig gene rearrangement, a variable (V) region is joined to a diversity (D) region and a joining (J) region. During the rearrangement process, random N-nucleotides (N) are inserted into the junctions to form a unique CDR3 sequence

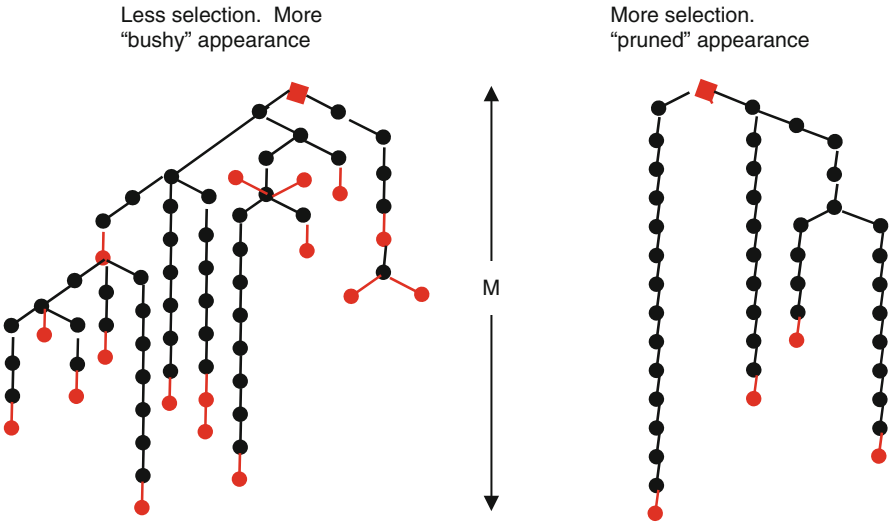

Fig. 3 Representations of lineage trees from clonal expansions of B-cells in the germinal centre reaction. Each node (round) represents one mutation away from the germline sequence (square). The shape of the lineage tree reflects the degree of selection acting on the clonal expansion as shown. The relative frequency of mutation in each lineage tree is compared by comparing the distances between the top and bottom of the lineage trees (M)

could result in the production of a population of cells with low affinity, such as is seen in the elderly, we investigated lineage trees from GC reactions in samples from patients of different ages for selection differences. We found a significant decrease in the degree of selection pressure acting on GC B-cells in the Peyer’s patch of the gut (but not the spleen). These data were confirmed by further analysis of the distribution of mutations within the Ig gene. A high level of replacement mutations in the complementarity-determining areas of the gene (relative to the more conserved Framework areas, Fig. 2) is expected in a selected Ig gene, and is indeed seen in the younger Peyer’s patch GC samples but not the old [114, 115].

An explanation for these apparent changes in selection is still elusive, but several factors could contribute. It may be solely a failure of the quality of B-cells in terms of specificity or signaling function. However, since FDCs and T-cells are important in the selection process they are also good candidates to investigate for the failure of selection pressure.

There is a well-documented age-related decline in thymus size and a reduced T-cell output. Homeostatic regulation in the face of reduced levels of naïve T-cells causes skewing of the T-cell repertoire which may reduce the availability of appropriate T-cell help for B-cells. Immunohistochemically stained human tissue sections have illustrated changes in T-cell populations in B-cell follicles [36,102]. The CD8+ T-cell numbers decline with age resulting in an increased CD4+/CD8+ ratio. Since it is CD4+ cells that are important in the affinity maturation process the significance of these findings is not known. There is, as yet, no information on whether the GC-specific T helper cells (CD4+ CD57+) are changed with age. CD40 ligand on GC

T-cells interacts with CD40 expressed on B-cells and this relationship is critical to T-cell dependent activation of B-cell proliferation, memory formation and class-switch recombination in the GC. Aged CD4 T-cells in mice have shown reduced CD40L expression [116] and in these animals there is a decrease in IgG levels reminiscent of the decreased IgG production in response to influenza vaccination in humans [40,41].

It has been suggested that the function of FDCs declines with increasing age [101, 117, 118]. Defects may be intrinsic to the FDCs themselves, or may be a failure of the FDC-B-cell interactions. FDCs have Fc receptors (FcR) and complement receptors 1 and 2 (CR1 and CR2) on their surface which retain antigen as immune complexes [119], and these interactions are crucial for the signaling and activation of antigen-specific B-cells. The immune complexes coat the FDCs to form bodies known as iccosomes. Aged FDCs have been reported to produce few to none of these iccosomes [117]. This may be due to the apparent down-regulation of FDC-FcγRII expression by FDC-bound immune complexes demonstrated in the GCs of old mice [120]. The resulting decrease in immune complex retention and presentation to B-cells would lead to lowered B-cell activation in the GC.

Although there is clearly a role for accessory cell failure in the age-related changes in GC responses, changes intrinsic to the B-cell itself are also responsible. The key enzyme in affinity maturation of B-cells is Activation Induced Cytidine Deaminase (AID) which is directly responsible for both hypermutation of Ig genes and class switching. Class switch recombination, from IgM to either IgG, IgA or IgE isotypes, creates antibodies with the same antigen specificity but different effector functions (e.g. complement fixing, secretory, opsonizing). AID expression is regulated by the E2A-encoded transcription factor E47. It has been shown, in mice, that E47 and AID expression is reduced in old B-cells [121], and that this reduction is due to a failure in the CD40 signaling pathway (indicative of T-dependent interactions) and the BAFF signaling pathway (indicative of T-independent reactions) [122]. Preliminary results also suggested that there was a similar decrease of E47 and AID in human peripheral blood B-cells [121].

4.5 Diversity of the B-Cell Repertoire

Evidence from our lineage tree studies on individual GCs indicated that in some instances the founder B-cells of a GC may have already been mutated. This occurred more often in the older samples and led us to postulate that B-cells which have previously been through the affinity maturation process might be being re-used in subsequent immune responses. If the starting population of B-cells has already been modified in response to a different antigen, then its ability to effectively change to accommodate a new antigen may be compromised. This could partially explain the compromized selection noted above. Naive B-cells are characterized by their IgD expression, and memory B-cells are characterized by having mutated Ig genes and expressing CD27 on their surface. It has been shown in mice that the older B-cell

population is made up of a greater number of B-cells carrying mutated Ig genes—i.e. memory B-cells [123]. Observations of an increased number of CD27+ B-cells in humans concur with this [124,125]. A change in serum IgD, which may also reflect an increase in the proportion of IgD-, memory, B-cells, has also been noted [84]. It is now well established, in mice, that naïve B-cell output into the periphery decreases with age [see p. 395 Scholz et al.]. There is, as yet, no evidence that human bone marrow B-cell output decreases with age, although it is known that children reconstitute B-cell function after bone marrow transplants more rapidly than adults do [126]. Therefore, if the overall number of B-cells is not drastically reduced, and there are less naive cells being produced, an increased proportion of memory B-cells is a logical conclusion [127]. Since B-cell memory appears to be maintained by proliferation [125] it is possible that proliferating memory B-cell clones make up for any shortfall in immunological space caused by lower naive B-cell input. However, a decrease in the number of memory B-cells with age has also been reported [128], so it would seem that this issue is still not completely resolved.

Our postulation, that GC reactions in the older samples were using "second hand" B-cells, lead us to further investigate B-cell diversity. A diverse and functional repertoire of antibodies is essential to produce an effective humoral immune response. If the repertoire of B and plasma cells is reduced, then the ability to recognize foreign antigen is severely compromised. B-cell diversity and antibody specificity are defined during the early stages of B lymphocyte differentiation, where the Ig genes are formed. The remarkable way in which gene segment rearrangement forms a complete Ig gene from different segments (Fig. 2) results in millions of different B-cells, each with a unique Ig sequence capable of producing antibody with distinctive specificity. Briefly, the Ig molecule consists of both heavy and light chains. There are three types of gene segments, variable (V), diversity (D, heavy chain only) and joining (J). The segments are randomly recombined to generate a V(D)J for the heavy chain (Fig. 2) or VJ for the light chain. Thus a germline repertoire of just 165 different V,D or J genes can result in a possible 8,116 different gene rearrangements. Combination of the heavy and light chains results in a possible 2,643,840 combinations. The region where the junctions join together is further diversified by an incomplete joining process. Addition and deletion of nucleotides by terminal deoxynucleotidy transferase (TdT) activity at these joints leads to junctional diversity. The VDJ joining region of the heavy chain, the CDR3 region, is so highly variable that it can be considered to be a fingerprint for that particular gene and the B-cell (and its progeny) that carries it.

There have been a number of studies which have looked for an age-related change in diversity by investigating the gene segment usage in Ig genes. The studies vary in design (looking at specific gene families only, or at specific isotypes, or only in response to a particular challenge) which may account for some of the discrepancies between them. The earliest report is probably the most comprehensive in terms of VH repertoire, although limited in the number of different subjects used (five old and one young) [106]. They showed an increase in usage of certain IGHV genes, in particular of the IGHV4 family [106]. However, this has since been contradicted. In another sequencing-based study of the IGHV4 repertoire in elderly human tonsil

Kolar et al. did not find any change [99]. The IGHV repertoire has also been analyzed, in a small group of individuals, using a family-specific PCR-based approach. This showed consistency in the IGHV repertoire between samples of the same individual at time points 10 years apart [107]. IGHV family specific studies alone may not pick up functionally significant differences in the repertoire. Although the IGHV3 family usage in response to pneumococcal polysaccharide vaccination showed no overall difference between the elderly and young adults, there was a significant loss of focus in the elderly response as evidenced by a loss of oligoclonality [52]. Furthermore, in the same experiments, a difference in Ig light chain usage was observed [129].

Other studies of B-cell diversity have concentrated on the CDR3 region. This, being the most variable region of the gene and having importance in antigen binding, has traditionally been an area used to define monoclonality and oligoclonality in pathology [130]. However, due to the cumbersome nature of sequencing and identifying V-D-J regions, the numbers of patients studied have generally been low, or limited to particular subsets of genes. For example, one study by Xue et al. [131] looked at D and J

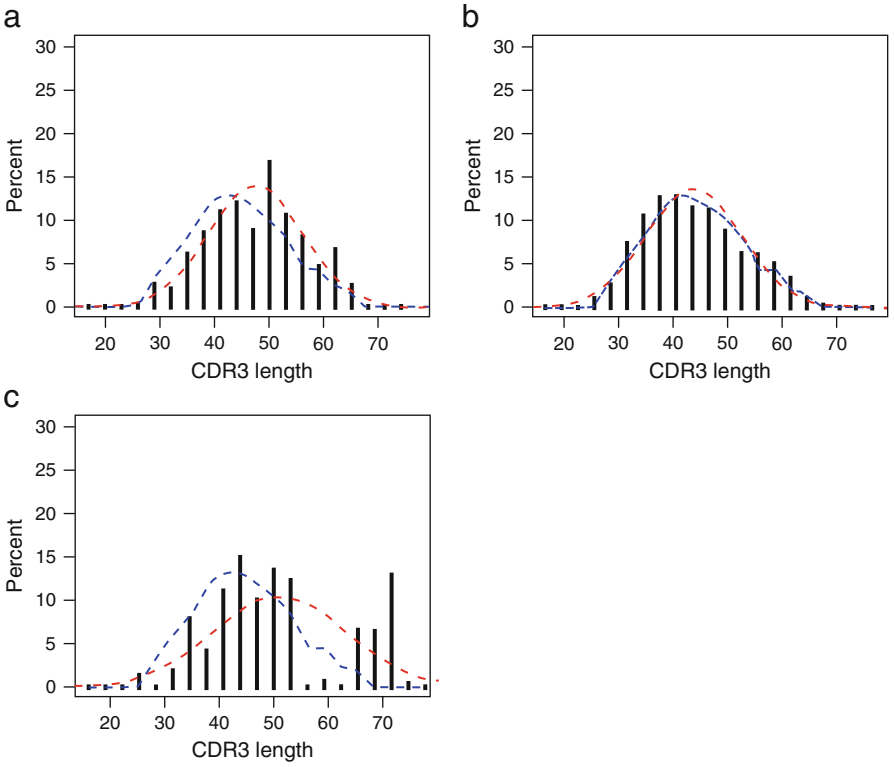

Fig. 4 Three different spectratype profiles. All are from old individuals (>88 years). Black bars represent the percentage of cdr3 regions of each different length. The red line represents the mean distribution for the young controls. The blue line shows the best fit for the individual sample data shown. A shows some B-cell repertoire restriction, B shows normally distributed cdr3 lengths, C shows an individual with very restricted B-cell repertoire. Approximately one-third of the old blood samples analyzed show restricted IgH repertoire along the lines of the spectratype in C

region usage as determined by sequencing and found no difference between younger and older samples. However, they had only seven young and seven old samples and only studied the CDR3 regions of IGHV5 family IgM genes. A more tractable method of looking at CDR3 diversity was also employed by them, using PCR to amplify all CDR3 regions and look at the spread of different sized fragments. This method of spectratyping has also been used in the analysis of T-cell repertoires [132–134] and enables the study of a much greater number of samples. We performed B-cell spectratyping on samples from peripheral blood of 33 old and 24 young subjects. The old samples are from the Swedish NONA Immune Longitudinal Study [135], from patients over 86 years of age. Preliminary data has shown that the B-cell repertoire is indeed restricted in a subgroup (approximately one third) of older people (Fig. 4).

4.6 Association of Monoclonal B-Cell Expansions With Age

Skewed B-cell spectratypes of the kind we have observed may have a number of aetiologies. It may indeed be true that a decreasing naïve B-cell output in the face of homeostatic mechanisms to keep the total number of B-cells the same has resulted in the repertoire being increasingly made up of antigen-experienced expansions of cells. Alternatively there may be pathological monoclonal expansions of B-cells, such as are seen in leukemia or lymphoma. Usually, these are diagnosed conditions, and individuals with this sort of medical history are excluded from studies on B-cell diversity. However, it might be possible that a pre-clinical condition exists in some people. An increase in monoclonal expansions of B-cells, both of CD5+ and CD5- phenotype, has previously been reported in older people [136]. Monoclonal gammopathy of undetermined significance (MGUS) is a predominant plasma-cell disorder [137] and has been shown to increase with age in both humans [137,138] and mouse [139]. It is characterized by an increase in presence of serum monoclonal Ig. MGUS is not found in young subjects, is prevalent in around 2% of over 50s and has been reported to vary in the elderly from 11% to 38% [138,140]. There is an association between MGUS and onset of multiple myeloma or related malignant condition with average risk assessed at about 1% per year [141]. Questions still remain as to what significance these populations have in the aging human. Obviously there is the possibility that MGUS accounts for some of the observed repertoire restriction with increasing age. However, our data does not suggest a high prevalence of such monoclonal expansions, and the restricted repertoires often have a more oligoclonal appearance.

5 Summary

We have outlined the different factors that are involved in making and maintaining an effective humoral immune response and how these may be affected by increasing age. It is clear that the ability to produce high affinity antibody with age is diminished

but there are many possible explanations as to why this might be. We have identified the most likely areas as being a decrease in the ability to select B-cells producing high affinity antibodies, and a decrease in the available repertoire in the first instance.

Many of the studies on B-cells in old age are carried out in mice and the data in humans is sadly lacking. Hopefully this situation will change in the future and maybe the advent of the use of B-cell depletion therapies for the treatment of autoimmune disease can help provide more human data on B-cell dynamics in individuals of different ages.

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Neutrophils

Neutrophil Granulocyte Functions in the Elderly

Peter Uciechowski and Lothar Rink

Contents

Abstract: The immune response weakens during aging. Especially, the altered functions of the lymphocytes of the adaptive immune system have been extensively studied. Aged persons >65 years display a predisposition to inflammation and infection combined with an increase in morbidity and mortality than younger individuals. In the past few years it has been discovered that certain functions of the innate immune system, which build the first line of defense against pathogenic microorganisms, are altered with aging. Among the cells of the innate immune system, neutrophilic granulocytes (polymorphonuclear leukocytes, PMN, neutrophils) eliminate invaded bacteria and fungi and play an accepted important role in regulation of the immune response. In vitro studies demonstrate that neutrophilic functions such as phagocytosis, generation of reactive oxygen species (ROS), intracellular killing, degranulation, and possibly chemotaxis are changed in elderly persons whereas the number of circulating neutrophils are unaltered compared to young persons. However, the reported data of different investigators regarding the above-mentioned functions are sometimes controversial. This may result from the use of different isolation methods of neutrophils, the degree of contaminating cells and preactivation of neutrophils during isolation. It could be shown that most of the adhesion surface molecules and receptors of neutrophils are not impaired in function and expression with age. But there is increasing evidence that age-related changes affect receptor-dependent signal transduction and membrane content and fluidity, which in turn lead to a decline in function and in inhibition of apoptosis. Further research

L. Rink (✉) · P. Uciechowski
Institute of Immunology
RWTH Aachen University Hospital
Pauwelsstr. 30
52074 Aachen, Germany
Tel.: +49 241 80 80208
Fax: +49 241 80 82613
E-mail: lrink@ukaachen.de

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has to be done to identify the molecular mechanisms that are responsible for the age-related modulations in human neutrophils.

Keywords: Adhesion • Polymorphonuclear leukocytes • Neutrophils • Phagocytosis • Chemotaxis • Degranulation • Intracellular killing • Inflammation • Apoptosis • G-CSF • GM-CSF • fMLP • IL-1RA • IL-1β • IL-3 • IL-8 • TNF-α • Toll-like receptor • MAPK • MyD88 • IRAK • p38 • ERK1/2 • Membrane fluidity • CD62L

1 Introduction

Elderly persons are more susceptible to microbial infections with an increase in morbidity and mortality due to declining immune status, termed immune senescence. In general, age-related changes include a decreased response to vaccination, increased incidence of inflammatory and autoimmune diseases and cancer. There are many efforts to clear up the molecular and cellular changes surrounding immune system dysfunctions. However, other factors such as nutrition, fitness, social components and diseases influence immunity of elderly persons making it difficult to detect single, age-dependent changes. To exclude those factors, the SENIEUR protocol was created to clearly separate age-related from nonage-related alterations of the immune system [1, 2]. This protocol sets the criteria in order for a healthy elderly person to participate in immunogerontological studies. The effects of aging are well-documented for the adaptive immune system, e.g. the alterations in T-cell count, phenotype, and function as well as reduced ability of B-cells to synthesize high affinity antibodies. But in the meantime, the importance of the innate immune system in fighting invading microorganisms and the cooperation with the adaptive immune system to ensure optimal immune response has become more widely accepted. Neutrophils display alterations of function, surface molecule expression, apoptosis and signal transduction with aging. These changes and their effect on the attenuation of neutrophil functions will be summarized and discussed by reviewing the literature.

2 Neutrophils

Polymorphonuclear leukocytes (PMN or neutrophils) are key effector cells of the innate immune system. They are the first cells to migrate rapidly to sites of infection and recognize and engulf microorganisms by phagocytosis. Neutrophils destroy and degrade invaded pathogenic bacteria and fungi via the release of reactive oxygen species and antimicrobial and proteolytic granule proteins, which are delivered to the phagosomes and to the extracellular environment. Additionally, neutrophils produce chemokines and cytokines that recruit and regulate the inflammatory response of macrophages, T-cells and neutrophils themselves. Finally, activated neutrophils

initiate an apoptotic programme where they are digested by macrophages without causing tissue damage and necrosis and therefore support the resolution of the inflammatory response [3].

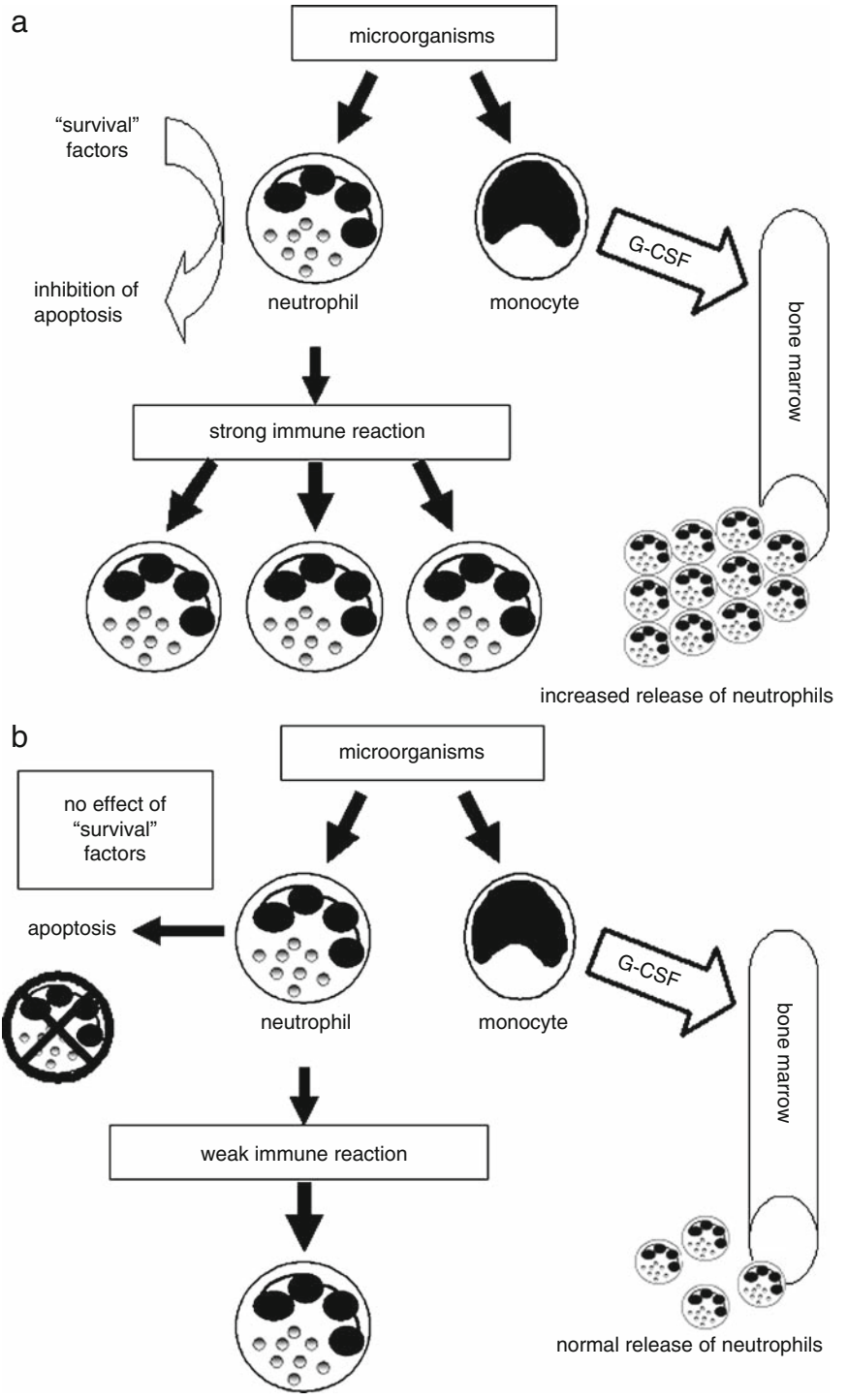
Neutrophils are short-lived cells and die by apoptosis spontaneously within 12–24 h of their release from the bone marrow. The adult bone marrow has to produce $1–2 \times 10^{11}$ neutrophils per day to sustain a sufficient cell number to efficiently fight infections [4]. This continuous production is controlled by granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3). To maintain the function of neutrophils summoned to infected tissue, "survival" factors such as lipopolysaccharide (LPS), hypoxic environment, complement and pro-inflammatory cytokines counteract apoptotic programs in neutrophils (Fig. 1) [5–8]. To fulfill their tasks in the defense against bacterial and fungal infections specific functions are regulated by specific receptors. These receptors are formyl-methionyl-leucyl peptide (fMLP), GM-CSF, complement, IgG Fc and interleukin-8 (IL-8) receptors [9]. Additionally, pattern recognition receptors, e.g. toll-like receptors (TLR), binding conserved molecular structures of most microorganisms, participate in the inflammatory response of PMN and other cells of the innate and adaptive immune system [10].

Historically, the role of neutrophils and their immune response has been underestimated and their function has been reduced to being only phagocytic active cells. In the past few years the views on the ability of neutrophils to bridge and regulate innate and adaptive immune responses have been shifted [11]. Using an isolation method to acquire highly purified human neutrophils without preactivation it was shown that neutrophils synthesize only a limited pattern of cytokines released, mainly IL-8, after stimulation [12, 13]. In addition, neutrophils produce large amounts of the antiinflammatory interleukin-1 receptor antagonist (IL-1RA) after stimulation or after high accumulation of neutrophils [14, 15]. Interestingly, neutrophils do not synthesize proinflammatory cytokines such as interleukin-1ß (IL-1ß), tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6) as described by others [16], which may be explained as a result of monocyte contaminations in the PMN isolates. Therefore, neutrophils not only recruit other immune cells to sites of infection but are also able to create an antiinflammatory environment that helps resolve inflammation.

3 Neutrophils and Aging

It is well-known that aging results in a predisposition to inflammation as well as to infections, which is associated with higher rates of mortality and morbidity [17, 18].

One might assume that impaired defense against invading pathogens such as fungi and bacteria is accompanied by a reduced amount of neutrophils as seen within the T- and B-cell system of elderly persons. But there are no alterations in the number of precursor cells in the bone marrow or of circulating neutrophils [19, 20]. Moreover, neutrophils have been described to be significantly increased in the aged [21]. Neutrophil precursor cells show a reduced proliferative response to G-CSF only (Fig. 1),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whereas responses to GM-CSF and IL-3 are not affected [19]. Elderly persons also display a normal neutrophilia during infection [22], indicating that GM-CSF and IL-3 mediate sufficient neutrophil production. However, loss of apoptotic rescue and a normal recruitment of neutrophils by G-CSF during infection might promote an impaired immune response with age (Fig. 1). In the case of severe chronic infection, neutropenia can be observed in the elderly, suggesting that persistent infection in the elderly impairs neutrophil recruitment [20].

4 Function

Although neutrophil count is elevated and adherence to endothelia is unchanged in elderly persons, impaired neutrophilic functions can be seen including a decline in phagocytic capacity in healthy elderly individuals accompanied by reduced intracellular killing [22–24]. This decline in function may contribute to increased susceptibility to bacterial infections in the elderly population. In contrast, aged persons fulfilling the SENIEUR criteria who also exhibit elevated numbers of granulocytes are functionally normal [25–28].

Studies that analyze phagocytosis of opsonized bacteria or yeast and opsonized zymosan by neutrophils have all demonstrated a significant impairment in phagocytic function in the elderly [29–33]. Additionally, the antibody-dependent phagocytosis mediated by Fc-receptors is also decreased [33]. Interestingly, the functions of these receptors are not changed, immunoglobulin and complement levels are normal and serum from elderly donors opsonize bacteria normally so that phagocytosis itself is impaired [32–34]. Butcher et al. [33] have shown that one of the receptors involved in recognizing antibodies on the surface of bacteria, CD16 (FcγRIII), is significantly reduced with age and may contribute to the observed decline in neutrophil phagocytic function with age [33].

After phagocytosis of pathogenic microorganisms, the phagosomes fuse with lysosomes containing bactericidal substances and build the phagolysosome. Therein the pathogen will be intracellularly killed. Besides other destructive components contained within the phagolysosome, intracellular killing is dependent on the generation of ROS, termed respiratory burst. This respiratory burst causes production of superoxide, hydrogen peroxide, and hypochloric acid, which are all toxic to microbes. Contradictory findings describing the respiratory burst after fMLP stimulation in neutrophils of the elderly have been reported. Some groups determined decreased respiratory burst activity after either fMLP [35–38], GM-CSF, or LPS stimulation [39, 40]. Wenisch et al. [32] showed a significant reduction in generation of ROS after stimulation with

Fig. 1 Recruitment and apoptosis of neutrophils during infection
a) After phagocytosis of invading microorganisms the apoptosis of neutrophils in young individuals is blocked via the release of survival factors. Additionally, G-CSF induces the release of a large number of neutrophils from the bone marrow leading to physiological neutrophil leukocytosis.
b) In elderly persons the inhibition of apoptosis of neutrophils is impaired and the recruitment of neutrophils from the bone marrow is not enhanced. This might result in an exhaustion of neutrophils and consequently lead to a reduced immune response with age.

Staphylococcus aureus (*S. aureus*) in contrast to no reduction after stimulation with *Escherichia coli*. These results are in concordance with the reported reduced ability of elderly to fight infections caused by gram-positive bacteria [41], since *S. aureus* frequently causes postoperative sepsis in the elderly.

Others studies using SENIEUR selected persons could not detect a difference in respiratory burst compared to younger persons even after stimulation with fMLP [22, 23]. The application of different stimuli led to various results, based on the assumption that distinct pathways of neutrophilic activation are involved. An early report by Tortorella et al. [42] showed that signal pathways may be impaired. Neutrophils obtained from elderly humans and stimulated with GM-CSF displayed a significant reduction in phosphorylated ERK1/2 levels and an even larger decrease in ERK1/2 activation. No changes in GM-CSF-induced p38 MAPK phosphorylation were observed [42]. This coincides with Larbi and colleagues reporting that p38 signaling is not involved in GM-CSF delayed apoptosis in any age-groups [43]. There are few reports about intracellular killing of fungi and bacteria in elderly people. They described that the capability of stimulated and unstimulated neutrophils to destroy *Candida albicans* is reduced by 10–50% in the elderly, and *E. coli* killing is 44% lower than that of young persons [39, 44]. The reason for impaired intracellular killing in neutrophils of the elderly is not clear yet. Although Piazzolla postulated that cytoskeleton affecting compounds are responsible for the alteration of fMLP stimulated superoxide generation [45], this does not illuminate the selective discrimination of one stimulant against the other. It is possible that triggering various signal transduction pathways after recognition of the pathogen and consequent activation of the neutrophil are responsible for an impaired defense towards one pathogen whereas the response to another remains unaltered in the elderly.

Neutrophils respond to various chemotactic products released either by the host or by the invading organism [46, 47]. Chemotaxis results from the initial contact and adhesion of PMN to endothelial cells through cell adhesion molecules, followed by migration through the endothelium following a chemotactic gradient to inflamed sites. Some investigators reported that chemotaxis remains largely unaltered in the elderly [34] or at least display a normal reaction after stimulation with fMLP [22, 23]. Other research groups found impaired chemotaxis when using other chemotactic substances and complement [32, 48, 49]. The consequence of the latter is that a fast recruitment to sites of infection is functionally restricted. That might explain the occurrence of severe wound infections by elderly persons since small numbers of pathogens cannot be efficiently eliminated. Corberand et al. [50] reported significantly decreased chemotaxis in people over the age of 80 years, and no significant difference in 60- to 70-year-old compared with young persons. Curiously, Niwa et al. [49] presented contrary results. They found a correlation between 60- and 70 year-old volunteers with diminished PMN chemotaxis and respiratory burst and mortality 7 years after the initial study. No difference between people older than 80 years old and the young could be seen. The explanation they offer was that there was no difference between the over-80-year-old persons and the younger ones because individuals with the more suitable neutrophils survived into the oldest age group [49, 50]. Similar data have

been obtained for degranulation and superoxide production in response to stimulants such as fMLP [31, 38].

5 Apoptosis

Apoptosis is involved not only in differentiation, development of tissue and homeostasis, but also in neurogenerative and immune diseases and cancer. Neutrophils display a fast apoptotic rate in vitro as well as in vivo. Apoptosis has to be well-balanced to ensure their survival and production; if the balance is shifted, the risk of chronic inflammatory diseases is enhanced.

The regulation of apoptosis of neutrophils is important to maintain longer survival in inflamed tissue or the resolution of inflammation. Without stimulation, the susceptibility of neutrophils to apoptosis is either slightly increased in the elderly or unaffected by aging [51–53].

It has been shown that the functions and the rescue from apoptosis by survival factors G-CSF, GM-CSF, IL-2 and LPS of PMN diminish with aging. In comparison to younger persons only GM-CSF alters apoptotic neutrophils slightly in the elderly [53]. Increased apoptotic rates of neutrophils at the site of infection might cause decreased bactericidal function (Fig. 1). DiLorenzo and coworkers reported a significant age-related decrease of formation of O^{2-} and chemotaxis whereas no significant correlation between age and the expression of the death receptor CD95 (APO1, Fas) on the granulocyte membrane could be detected. The authors suggest that an increase of CD95-mediated apoptosis of neutrophils might play a minor role in the impairment of neutrophilic function [54]. Fulop et al. [55, 56] investigated the role of antiapoptotic Mcl1 and pro-apoptotic Bax in decreased apoptosis inhibition in PMN of the elderly. The authors found that the expression of Bax was unchanged in elderly and young persons; also treatment with GM-CSF could not modulate the Bax expression. Similar results were obtained by examining Mcl1, which was upregulated after GM-CSF stimulation in young persons, whereas in the elderly no difference was found between stimulation and spontaneous apoptosis. By comparing the Bax/Mcl1 ratio after GM-CSF stimulation in younger and aged persons there was only a slight difference in the Bax/Mcl1 ratio in the elderly, whereas Mcl1 expression was increased relative to Bax in neutrophils from younger individuals. These findings indicate an important role of Bax and Mcl1 in the survival of neutrophils mediated by GM-CSF. The Janus tyrosine kinase (Jak)2-signal transducer and activator of transcription (Stat)5 signal transduction pathway is also modulated in elderly persons [44, 56]. Since Jak2 is related to the expression of antiapoptotic Bcl-2 there might be a possible link between Jak2 and Mcl1 being involved in the decreased rescue of neutrophils from apoptosis (Fig. 2) [56]. Larbi et al. [43] presented evidence that a modulation in the p42/p44 (ERK1/2) mitogen activated protein kinase (MAPK) activation occurs in PMN of elderly subjects under GM-CSF stimulation and is in part responsible for the decreased apoptotic decline of PMN in the elderly. This might be the reason why GM-CSF was not able to down-regu-

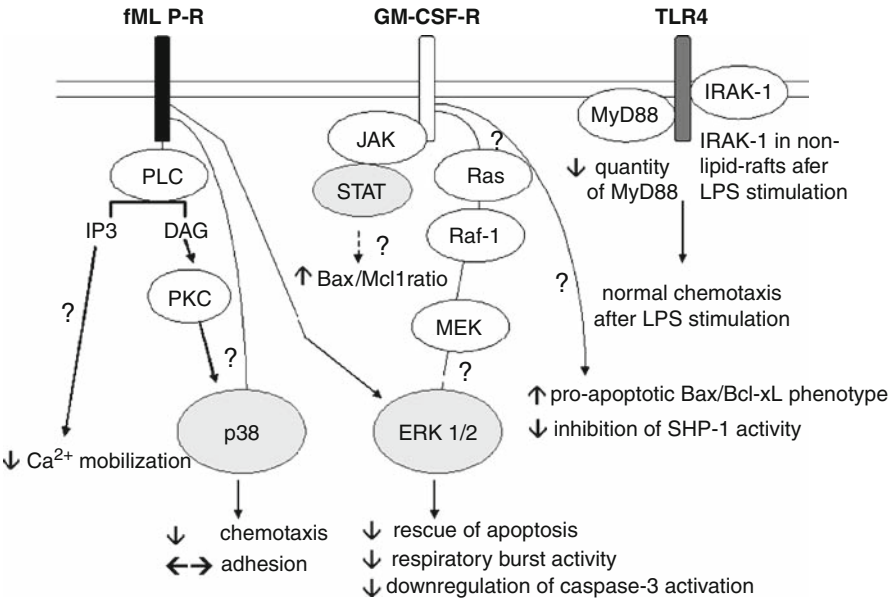

Fig. 2 Signaling in neutrophils of the elderly
Age-related impairment in intracellular signaling after binding of the appropriate ligands to their receptors leading to altered functions of neutrophils. Question marks display defects in different signal pathways associated with age (modified and adapted from Fulop et al. [56]). PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol triphosphate; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase; PKC, protein kinase C.

late caspase-3 activation in neutrophils of elderly persons. Interestingly, the authors observed that GM-CSF changed the proapoptotic phenotype to an antiapoptotic phenotype by alteration of the bcl-2 family members Bax and Bcl-xL in young neutrophils in an MAPK independent way whereas this could not be seen in aged neutrophils [43]. Taken together, these modulations might be responsible for the creation of a proapoptotic environment and could explain the increased incidence of infections in the elderly (Figs. 1, 2).

6 Signal Transduction

Activation of the fMLP receptor via phospholipase C (PLC) leads to the production of diacylglycerol (DAG) and inositolphosphate 3 (IP3), the latter initiates the enhancement of intracellular Ca^{2+}. DAG induces the membrane translocation of protein kinase C (PKC) and phosphorylation of MAPK family members. Intracellular Ca^{2+} is decreased in stimulated neutrophils from elderly persons (Fig. 2), suggesting that there is an impairment in Ca^{2+} flux during cell signaling [53, 56–59]. Interestingly, resting neutrophils of elderly subjects show an enhanced level of intracel-

lular Ca^{2+} [32, 57, 60]. Preactivation, modulation of the aged plasma membrane followed by altered receptor and adapter protein linkage and defects in the early phase of signal transduction might lead to the impairment of Ca^{2+} mobilization of aged neutrophils after fMLP stimulation. By investigating the impaired Ca^{2+} mobilization in aged neutrophils, Klut et al. [61] found heterogeneity of the examined neutrophils concerning time and magnitude of the response. A reduced number of neutrophils in the elderly were able to generate an effective reaction, hinting at a possible subpopulation [61].

After fMLP stimulation, PKC might also activate the p38 signal pathway, which is involved in regulating gene transcription, chemotaxis and adhesion. The ERK1/2 signal pathway is also triggered after fMLP stimulation playing a role in adhesion and respiratory burst activity. Defects in the signal cascades of both pathways and the decrease in activation and phosphorylation levels of p38 and ERK1/2 MAPKs are suggested to affect impaired neutrophilic functions in the elderly (Fig. 2) [56].

GM-CSF is able to activate the JAK/STAT pathway, the Ras-Raf-1-MEK-ERK1/2 pathway and phosphatidyl-inositol 3 kinase (PI-3K) triggered signaling [56]. Investigating the role of protein tyrosine phosphatases (PTP), especially Src homology domain-containing protein tyrosine phosphatase-1 (SHP-1), Fortin et al. [62] suggested a differential effect of GM-CSF on phosphatase activity in modulating neutrophil functions with aging. SHP-1 is a negative regulator of signal transduction and can negatively regulate Src kinases, such as the Jak or Lyn kinase, elicited by GM-CSF in PMN. When recruited to the plasma membrane and activated, SHP-1 dephosphorylates proteins activated by receptors, and inhibits cell activation. The authors could show that SHP-1 phosphatase activity cannot be down-regulated after short stimulation with GM-CSF in the neutrophils of the elderly persons in contrast to neutrophils of young. In lipid rafts from neutrophils of elderly, SHP-1 is continuously present, whereas in the neutrophils of young donors, SHP-1 is rapidly dissociated after stimulation by GM-CSF and is recruited back during a longer period of stimulation. In contrast to younger persons, SHP-1 is constantly recruited to Lyn, which cannot be relieved by GM-CSF. These modulations together with the above-mentioned changes in the Jak2-Stat5 and ERK1/2 signal pathways might contribute to the decreased GM-CSF effects on neutrophils [62]. Fig. 2 summarizes the effects of aging in signal transduction.

7 Adhesion, Surface Molecules and Receptors

After receiving a chemotactic signal, the rolling neutrophil adheres via integin molecules to endothelial cells and migrates through the endothelium (diapedesis) towards the site of infection. Adhesion appears not to be impaired in the elderly. After stimulation with fMLP, zymosan, phorbol myristate acetate (PMA), or calcium ionophores, human neutrophils from young and elderly persons displayed no difference in adhesion to plastic, gelatin, and bovine aortic endothelium [37, 44].

Additionally, a normal or enhanced adherence of neutrophils to endothelia or thrombocytes has been described, but it is not clear whether this has an effect on increased tissue migration in vivo. One might argue that increased adherence is caused by slightly enhanced expression of CD15 (Lewis X) and CD11b (Mac-1, complement receptor 3) on neutrophils [31]. In contrary, no increase of CD11b and CD15 but a decrease of CD62L (L-selectin) was observed by others [33, 63]. Interestingly, the expression of the other two integrins, CD11a (leukocyte function antigen, LFA-1) and CD11c (p150, 95) involved in cell adhesion, is not affected [22, 23, 31, 33].

De Martinis et al. [64] compared the expression of CD50 (ICAM-3; a ligand for CD11a/CD18) and CD62L adhesion molecules in peripheral blood granulocytes and monocytes between healthy elderly and young persons. They found a decrease in the percentage of granulocytes and monocytes expressing CD62L in the elderly but no alteration in the density expression on both cell types suggesting a preactivation which might contribute to the proinflammatory status in aging. The authors described a downregulation of the density expression of CD50 at a per cell level on granulocytes and a decrease of CD50 density expression on monocytes but an expansion of CD50 positive cells in elderly persons. This indicates that the loss of CD62L on granulocytes leads to impairment in cell adhesion and likely contributes to the enhanced susceptibility to acute infections in elderly persons.

Noble et al. [65] observed a significantly lower recruitment of early activation marker CD69 from the vesicles to the plasma membrane after stimulation with PMA in elderly people (fulfilling the SENIEUR criteria) than in younger persons. fMLP in contrast had no influence in different expression of CD69 in young and elderly persons, suggesting again the impairment of distinct pathways within aging. Interestingly, also the CD69 expression in natural killer (NK) cells is decreased [66].

There is growing evidence that aging is accompanied by changes in receptor signaling pathways and membrane fluidity [22, 24, 37, 43, 56, 62]. In contrast to other cells the fluidity of the PMN membrane increases with age, caused by alterations in the cholesterol/phospholipid content of the membrane [56, 67, 68]. These modulations result in changed function of lipid rafts, which directly influence TLRs and GM-CSF signaling. Additionally actin, which may play a role in cell-surface receptor movement and expression, has been indicated to contribute to the changed ROS production [69]. In summary, these alterations in signaling may impair the effector functions of neutrophils in aging.

After stimulation, the fMLP receptor which is coupled to a Pertussis toxin-sensitive G protein induces the production of superoxide anion, hydrogen peroxide, nitrite oxide (NO) and an increase in intracellular free calcium. The influence of aging on the release of free radicals has been investigated by different laboratories for a long period of time (reviewed by Ref. 24). Some investigators reported a decreased synthesis of free radicals by neutrophils of elderly persons, but found no change in the expression of fMLP receptor number, [37, 38, 56] whereas others could not confirm those data [22]. A recent study by Fulop et al. [56] examining

neutrophils isolated from young and aged persons who met criteria defined by the SENIEUR protocol, showed a significantly lower production of superoxide anion under fMLP stimulation and/or GM-CSF priming in PMN from elderly persons compared with younger ones. Fulop et al. [56] postulate the existence of a subpopulation of neutrophils in aged persons, which seems to be responsible for a significantly higher superoxide anion production after 48 h when compared with younger PMN, although they found a reduced superoxide anion production after 24 h stimulation with fMLP and GM-CSF in elderly persons. The authors suggest that PMN from elderly persons might act heterogeneously to down-regulate responses to stimulation than PMN from younger persons, which react more efficiently.

Toll-like receptors belong to the family of pattern recognition receptors and have a specificity to bind substances consisting of conserved motifs of bacteria, fungi and virus. To date, ten different human TLR have been identified, including three intracellularly located types. After ligand binding, the central adapter molecule, myeloid differentiation primary response protein 88 (MyD88), transduces signals into the cell by recruiting a cascade of serine–threonine kinases and IL-1 receptor-associated protein kinases (IRAKs), leading to nuclear factor kappa B (NF-kB)-dependent transcription of proinflammatory genes. Although there is a MyD88 independent way, stimulation via TLR leads to the release of pro-inflammatory cytokines such as interleukin-1, IL-6 or TNF-α. The additional production of chemokines and upregulation of surface molecules through TLR signaling build a bridge between innate and adaptive immune responses. Few reports about the influence of age on TLR exist at present. Renshaw et al. [70] described that LPS (ligand for TLR4, gram- bacteria)-stimulated macrophages from aged mice synthesize less IL-6 and TNF-α than younger ones. This study was confirmed by Boehmer [71]. Additionally, a lower TLR4 mRNA level compared with those of younger macrophages was found in aged macrophages by Renshaw et al. [70, 71], whereas others did not observe a variation in TLR4 surface expression with age. These results are not compatible with the situation in elderly human beings where elevated levels of circulating proinflammatory cytokines are generally observed; especially since elderly monocytes after LPS stimulation produce significantly higher amounts of IL-6 and TNF-α [24, 72, 73].

By studying the expression of TLR2 (ligand: components of gram+ bacteria) and TLR4, Fulop et al. [56] did not observe any changes in the proportion of neutrophils expressing TLR2/4 nor in the expression of both receptors on the surface of neutrophils. They also observed no differences of fMLP and GM-CSF receptor expression with aging [56]. What they found was an increase of TLR4 expression in unstimulated raft and nonraft fractions and no redistribution after LPS stimulation in elderly persons in contrast to younger individuals.

Although the TLR2 and TLR4 expression remains unchanged, one key component of the TLR signaling, IRAK-1, was not found to be associated with lipid-rafts after stimulation with LPS. Additionally, the main adapter protein of the TLR signal pathway, MyD88, was significantly reduced in the plasma membrane of elderly persons (Fig. 2). These observations confirm the thesis that age-related

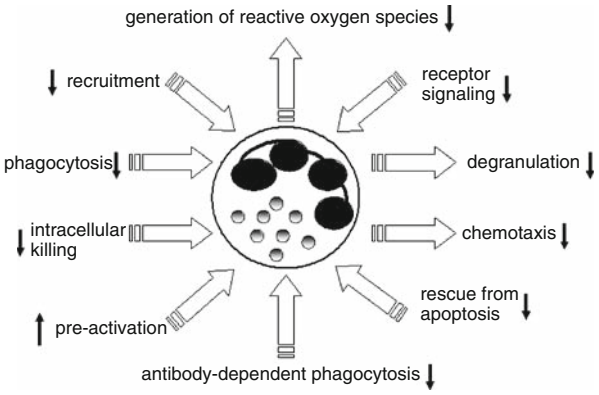

Fig. 3 Overview of impaired neutrophilic functions with aging

alterations influence receptor-driven signal transduction but do not explain normal LPS mediated chemotaxis of neutrophils of elderly persons. One might speculate that other signal pathways are involved or a nonreceptor-driven function of LPS might exist [56, 72].

The views regarding the importance of neutrophils in immune responses have been changed over the past few years. In immunogerontological studies, contradictory data may result from different isolation techniques of neutrophils, distinct amounts of contaminating cells, preactivation of neutrophils during isolation, and selection criteria of aged persons.

Taken together, the neutrophils are also affected through aging. The changes are found in decreased chemotactic functions which may be associated with the loss of CD62L. Therefore, CD62L-mediated migration might be hampered and this might lead to increased infection. The shedding of CD62L from the cell surface of neutrophils is also a sign of preactivation as postulated by other groups [17, 18] and conforms well to the observation of enhanced Ca^{2+} flux in elderly persons. Yet, one has to be cautious with regards to the purification process of neutrophils since some substances may cause a decrease in CD62L expression [12, 13, 24]. With the exception of CD62L, CD50 and CD16 other surface molecules such as CD11a, b, c/CD18 were not found to be modulated in aged persons when compared to younger individuals. Recent publications indicate a decline in signal transduction as being responsible for receptor-mediated responses and apoptotic rescue mechanisms. Additionally, altered plasma membrane content and fluidity of neutrophils in the elderly appear to influence signal transduction. It should be pointed out that different PMN isolation techniques and monocyte contaminations cannot be excluded as a possible explanation for the controversial results published by distinct groups of investigators [24]. The importance of purity and preactivation of PMN preparations in defining and differentiating PMN signals from those by others could be demonstrated recently [13, 14]. By investigating cytokine production of neutrophils in the elderly, one must also take into account that aged monocytes produce significantly more proinflammatory cytokines after stimulation than those of younger persons [72, 74].

Table 1 Age-related changes of neutrophils

Parameter/function	Stimulants/targets	Reported effect
Number of circulating neutrophils		←→19, 20 ↑21
Number of precursors in bone marrow		←→19
Proliferation of neutrophilic precursors in response to	G-CSF	↓19
	IL-3	←→19
	GM-CSF	←→19
Phagocytosis	Opsonized bacteria, yeast	↓29–33
	Antibody-dependent CD16-mediated	↓33
Respiratory burst	fMLP	↓ 35–38←→ 22, 23
	GM-CSF, LPS	↓39, 40
	Gram positive bacteria	↓32, 48
	Gram negative bacteria	←→32
Degranulation	fMLP	↓31, 38
Chemotaxis	fMLP, GM-CSF, LPS	↓32, 48–50←→22, 23, 34
Intracellular killing	Gram negative bacteria, fungi	↓39, 44
Adhesion	Endothelia, thrombocytes	←→37↗31
	CD11a-c/CD18	←→33, 63, 65↗31 (CD11b)
	CD15	←→63↗31
	CD50	↘64
	CD62L	↓64
Apoptosis		←→51, 52↗53
rescue by	IL-2, LPS, G-CSF, GM-CSF	↓51, 53
CD95 induced apoptosis; expression of CD95		←→54
down-regulation of caspase-3 activity	GM-CSF	↓43
Bax/Mcl1	GM-CSF	↑55, 56
Antiapoptotic phenotype	GM-CSF	↓43
inhibition SHP-1 activity	GM-CSF	↓62
Jak2-Stat5 pathway	GM-CSF	↓56
Signal transduction		↗42, 56
intracellular Ca^{2+} level		↑32, 57, 60
intracellular Ca^{2+} mobilization	fMLP	↓57–59
ERK1/2 MAPK pathway	GM-CSF	↓42
p38 MAPK pathway	GM-CSF	←→42, 43
plasma membrane fluidity	Receptor signaling in relation with lipid rafts	↓56, 67, 68
Expression of surface molecules		
	CD69	↓65
	fMLP-R	←→ 37, 38, 53↘22
	GM-CSF-R	←→ 56
	TLR2, 4	←→ 56

↓, decreased; ↘, slightly decreased; ↑, increased; ↗, slightly increased; ←→, unchanged

8 Conclusions

Although neutrophilic counts are normal or slightly increased in aged persons compared to young individuals aging influences the functional properties of neutrophils. The changes affect phagocytosis in neutrophils from elderly subjects where significant reduction along with decreased antibody-dependent phagocytosis was observed. Also, the other toxic mechanisms to destroy pathogenic microorganisms such as ROS generation, degranulation and intracellular killing, are impaired by age. Studies of chemotaxis have shown contrary results, so it has to be clarified if migratory responses of neutrophils from healthy, elderly persons are in fact altered. The decline in functionality, impaired Ca^{2+} mobilization and delayed rescue from apoptosis during aging appear to arise from defects of several signaling pathways, altered plasma membrane components and modulated protein tyrosine phosphatase activity. The molecular mechanisms responsible for those alterations in signal transduction and why distinctive stimuli cause different effects are still poorly understood. Fig. 3 and Table 1 summarize the age-related changes in neutrophils.

Further research is required since neutrophils display more features than formerly assumed, it would spread light on the deficiencies that occur during the aging process and could be beneficial to the elderly in the future.

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Signal Transduction Changes in fMLP, TLRs, TREM-1 and GM-CSF Receptors in PMN with Aging

Carl F. Fortin, Anis Larbi, Gilles Dupuis and Tamas Fulop

Abstract: It is well known that the immune response is decreased with aging leading to a higher susceptibility to infections, cancers and autoimmune disorders. The most widely studied alterations are relative to the adaptive immune response. Recently, the role of the innate immune response as first line of defence

T. Fulop (✉)
Research Center on Aging
Department of Medicine
Immunology Graduate Programme
Faculty of Medicine University of Sherbrooke
Sherbrooke, Quebec, Canada

C. F. Fortin
Clinical Research Center
Immunology Graduate Programme
Faculty of Medicine
University of Sherbrooke
Sherbrooke, Quebec, Canada

A. Larbi
Center for Medical Research
Section for Transplant-Immunology and Immuno-Hematology
Tuebingen Aging and Tumor Immunology Group
University of Tuebingen
Tuebingen, Germany

G. Dupuis
Clinical Research Center
Department of Biochemistry
Immunology Graduate Programme, Faculty of Medicine
University of Sherbrooke
Sherbrooke, Quebec, Canada

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against bacterial invasion and modulator of the adaptive immune response has been widely recognized. One of the most important cell components of the innate response is neutrophils. It is now accepted that neutrophil functions are changed with age however the degree of these changes is still debated. With aging there is an alteration of the receptor driven functions of human neutrophils, such as superoxide anion production, chemotaxis and apoptosis. One of the alterations underlying these functional changes is the decrease of the receptor signalling elicited by specific receptors. Alterations were also found in the neutrophil membrane lipid rafts. These alterations in neutrophils functions and signal transduction occurring with aging might contribute to the increased infections with aging.

1 Introduction

Neutrophils, also known as polymorphonuclear leukocytes (PMN), are the first cells to arrive at the site of an aggression (Medzhitov and Janeway, 2000). Their role is to eliminate the aggression in a non specific way to prevent ongoing tissue damage and in the mean time regulate and determine the adaptive immune response. They are very efficacious to combat the bacterial and fungal infections (Lehrer et al. 1988). The neutrophils are very short lived cells except if they receive a proinflammatory signal. These signals may prolong the survival of neutrophils to be more effective in eliminating pathogens. Neutrophil functions with aging are changing, mainly those of chemotaxis, free radical production and adherence. Most of these functions are mediated through the engagement of a receptor including formyl methionyl leucine peptide (fMLP), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-8 (IL-8) receptors (Fulop and Seres, 1994). Recently, novel class of receptors emerged and, they have profound impact on the functions of human PMN. Among them, the pattern recognition receptors (PRRs), including at least 10 toll like receptors (TLRs) which recognize conserved molecular structures, related mostly to pathogens, were described and extensively studied (Medzhitov 2001; Krishnana et al. 2007). Furthermore, the triggering receptor expressed on myeloid cells-1 (TREM-1) is a recent addition to the growing members of activating receptors that are members of the Ig superfamily and, is up-regulated at the surface of PMN and monocytes in infection and LPS-induced sepsis in mice (Bouchon et al. 2000; Bleharski et al. 2003; Gibot 2006). Over the past few years, it has been demonstrated that PMN-specific receptor-driven effector functions are altered with aging (Fulop et al. 1997; Varga et al. 1997; Fulop et al. 2004). One of the causes of these decreased functions could be the alteration of signalling with aging via various receptors of neutrophils (Fulop et al. 1985a,b; Vlahos and Matter, 1992; Wenisch et al. 2000; Lord et al. 2001; Schröder and Rink, 2003; Fulop et al. 2001; Biasi et al. 1996; Seres et al. 1993). This chapter will describe our present knowledge concerning the signal transduction pathways in neutrophils with aging elicited by fMLP, GM-CSF, TLR and TREM-1 ligands.

2 Neutrophil Function Changes with Aging

PMN are short-lived cells that play important roles in both host defence and acute inflammation. They represent the first line of defence against an assault. They are committed to die in circulation within 18 hours unless activated (Akgul et al. 2001). This activation results in the initiation of an inflammatory response leading to chemotaxis via adhesion to endothelial cells, migration and the development of effector functions such as free radical production (Babior 2000). The adhesion (Butcher et al. 2001) and migration (Biasi et al. 1996) functions of PMN were found unchanged with aging. Recent data on chemotaxis indicate a decrease during aging towards fMLP and GM-CSF as chemoattractants (Fulop et al. 2004). The inability of GM-CSF to prime PMN of elderly for superoxide anion production was also described (Seres et al. 1993). It is of note that the number of receptors involved in PMN chemotaxis has not been found to change with aging. We have demonstrated some time ago that the production of free radicals by PMN of elderly subjects was decreased under fMLP stimulation while the number of fMLP receptors did not change (Fulop et al. 1985a, 1989) and, this was also found by many laboratories (Braga et al. 1998; Biasi et al. 1996), while others found no changes (Lord et al. 2001). It is of note that the variations in PMN superoxide production with aging were dependent on the stimuli indicating different pathways of neutrophil activation. It was shown that gram positive pathogens induce a decreased production, while gram-negative ones induce no reduction (Wenisch et al. 2000). Insofar, these pathogens modulate PMN functions through different TLRs (Hayashi et al. 2003).Certain proinflammatory cytokines, or other molecules, were shown to prolong the life span and the functional survival of PMN (Whyte et al. 1999). Among these molecules are GM-CSF, LPS and IL-6. Other bacterial products such as fMLP, LPS, lipoteichoic acid modulate the effector functions of neutrophils. We and others have found that the PMN of elderly subjects can not be rescued from apoptosis by various agents known to be very effective for PMN of young subjects (Fulop et al. 1997; Tortorella et al. 1998, 2001).

Thus, we can hypothesize that alterations of the signal transduction pathways of the various receptors are involved in the altered neutrophil functions with aging (Fulop and Seres 1994; Fulop et al. 2001). This altered signal transduction can be related to changes in the physico-chemical properties of the PMN membrane with ageing determining its fluidity. It has been shown that changes in membrane fluidity affect PMN functions, such as chemotaxis, superoxide anion production (Yuli 1982; Alvarez et 2001). An age-dependent decrease in plasma membrane fluidity has been shown in various cell types (Rivnay et al. 1980; Shinitzki 1987) including T-lymphocytes (Larbi 2004a,b), whereas in neutrophils an increase was observed in the membrane fluidity (Fulop et al. 2004). These data suggest that either of these changes in membrane fluidity with aging could be deleterious for cellular functions. It should be remembered that PMN are very short lived cells in contrast to all the others studied, explaining the differential changes of membrane fluidity with aging. Very recently, the presence of lipid rafts in PMN cell membrane has been

described and an important role in PMN signal transduction has been suggested for them (Kandzelskii et al. 2004; Sitrin 2004; Fortin 2007b, c). Changes in membrane fluidity will affect the function of lipid rafts (Simons and Ehehalt 2002; Simons and Ilkonen 1997), which are special membrane microdomains for signalling that are playing an important role in cellular functions, including chemotaxis (Ibanez 2004). Thus, age-related changes in the cell membrane affect the membrane properties, which in turn determine the signal transduction leading to altered effector functions, such as chemotaxis, superoxide anion production and apoptosis. This might influence the sequence of all the other effector functions of PMN with aging. We will review herein some specific receptor signalling changes with aging.

3 Signal Transduction Changes and Lipid Rafts in Neutrophils in Relation to fMLP, GM-CSF and Toll-like Receptors with Ageing

There are rather few data concerning the signal transduction in PMN with aging as compared to those in T-lymphocytes (Larbi et al. 2004a). Nevertheless, accumulating data suggest that aging cause alterations of specific receptor signalling pathways in PMN (Fulop and Seres 1994; Fulop et al. 2004; Lord et al. 2001; Schröder and Rink 2003; Tortorella et al. 2007). The recent description of lipid rafts in PMN membrane will also rapidly improve our understanding on PMN signalling pathways and permit their extension to a better investigation of the PMN signal transduction with aging.

3.1 fMLP Receptor

Formyl peptides engage receptors that belong to the seven transmembrane G protein-coupled receptor (GPCR) family and trigger neutrophil responses, i.e., chemotaxis, up-regulation of surface receptors, release of proteolytic enzymes from granules and, ROS production (Varga et al. 1988; Mcleish et al. 1989; Varga et al. 1989; Rabiet et al. 2007). These responses are largely inhibited by Bordetella pertussis toxin, indicating that signal transduction is dependent on a heterotrimeric G protein of the Gi type. We will review current knowledge about the peptide-induced activation of chemoattractant receptors and their regulation, with special emphasis on the human formyl peptide receptor family (FPR, FPRL1, and FPRL2). Upon chemoattractant binding, receptors undergo a conformational change that enables them to interact with the Gi2 protein thereby triggering both the exchange of GDP to GTP in the G protein α subunit and, the dissociation of the $\beta\gamma$ complex from the α subunit (Gierschik et al. 1989). Following its dissociation from the α subunit, the G protein $\beta\gamma$ subunits activate the phospholipase Cβ2 (PLCβ2) (Camps et al. 1992) and the phosphoinositide 3-kinase γ (PI3Kγ) (Stoyanov et al. 1995). PI3Kγ

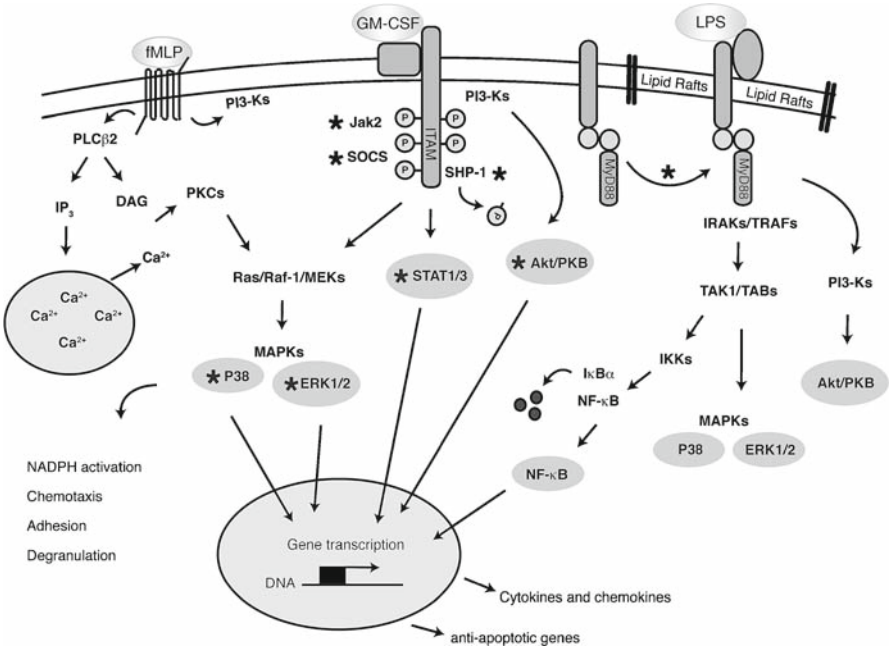

Fig. 1 Alterations in the receptor-mediated signal transduction in human neutrophils with aging. In neutrophils, fMLPR engagement leads to activation of PI3-K and PLCγ2, this in turn leads to the production of DAG and the influx of Ca^{2+} to the cytosol where they activate the PKCs. The PKCs activate the downstream MAPKs P38 and ERK1/2 through Ras. The ligand of GM-CSFR, for its part, induces the phosphorylation of residues in the ITAM of the common β-chain These events will induce the recruitment of various signalling molecules to the GM-CSFR and, will lead to the activation of the MAPKs P38 and ERK1/2, the Jak2-STAT1/3 and the PI3-K-Akt/PKB signalling pathway. The down-regulation of the GM-CSFR is mediated by phosphatases, like SHP-1, that removes phospho-groups on the ITAM, or the SOCS family of protein, which bind Jak2 and other activating upstream kinases thereby impeding the recruitment of signalling molecules on the receptor. Upon engagement, TLR4 is recruited into lipid rafts and, with the accessory molecule MyD88, leads to the activation of the MAPKs P38 and ERK1/2 and the transcription factor NF-κB through the IRAK/TRAFs and TAK1/TABs complexes. It also activates the PI3-K-Akt/PKB pathway through largely unknown mechanisms. The downstream kinases and the transcription factors elicited by these receptors mediate, in the cytosol and in the nucleus, the functional responses of human neutrophils such as respiratory burst, chemotaxis, degranulation and production of cytokines and chemokines. The asterisks indicate impairment that have been found in the signalling pathways of these receptor with aging in human neutrophils. One can appreciate the work that remains to be done as the absence of asterisk indicates that potential alterations were not studied in the elderly for these signalling molecules

converts the membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which is required for both the directed migration of neutrophils in a gradient of fMLP and the generation of superoxide mediated by the stimulation of chemoattractant receptors. The activation of PLCβ2, which induces the production of IP_3, leads to an increase of intracellular free calcium and of DAG, which result in the translocation of PKC to the membrane and,

leading to the phosphorylation of members of the MAPK family (Chang and Wang 1999). Neutrophils express the classical PKC isoforms (α, βI, and βII), the novel PKC isoforms δ and the atypical PKC isoforms ζ. The activation of PKC isoforms play a role in the regulation of NADPH oxidase activity. The extracellular signal-regulated kinases (ERK1/2) and the stress-activated p38 MAP kinase are activated by chemoattractants in neutrophils. These two signalling pathways are thought to participate at different degrees in adherence, chemotaxis and superoxide production. PLA_2-α is phosphorylated by MAP kinases and is translocated to the plasma membrane in a calcium-dependent manner where it produces free fatty acids and lysophospholipids.

Stimulation of the cells by fMLP induces, via the production of IP_3 and the opening of calcium channels in the membrane, an increase in intracellular free calcium. This increase is normally very rapid and returns to the prestimulation level relatively quickly. There is a slight difference between young and elderly subjects in the intracellular free calcium kinetics stimulated by fMLP in PMN (Biasi et al. 1996). The amount of the intracellular free calcium inside the cells is higher under fMLP stimulation in PMN of young than elderly subjects, while it was higher in the PMN of elderly at unstimulated status (Fulop and Seres 1994). This indicates a slight activation status of PMN with aging due to the low grade inflammation occurring with physiological aging (Franceschi et al. 2000; Meyer et al. 1998). The return of the intracellular free calcium must be tightly regulated, because if it remains high this could lead to cell death via the activation of certain intracellular proteases such as calpains or endonucleases. These data indicate that aging is associated with a decrease in the early phase of signal transduction in PMN.

The induction of PKC via the ras pathway in turn induces the activation of MAPK family members when the PMN are stimulated by fMLP (Zu et al. 1998). MAPKs are a family of serine/threonine kinases that are activated by a cascade of protein kinase reactions (Kyriakis and Avrach 1996), which are not completely elucidated in human neutrophils, even after fMLP stimulation. In rat neutrophils the activation of Lyn is associated with binding to the Shc adaptor protein and allows the G protein-coupled receptors to modulate the activity of the Ras/ERK cascade (Chang and Wang 1999). Nevertheless, investigations of human neutrophils have suggested that p38 MAPK is involved in an intracellular cascade that regulates stress-activated signal transduction. The p38 MAPK can phosphorylate transcription factors, thereby regulating gene expression and, can also phosphorylate other proteins to stimulate NADPH oxidase activity, adhesion and chemotaxis (Kyriakis and Avreach 1996; Zu et al. 1998; Heuertz et al. 1999; Yagisawa et al. 1999; Chang and Wang 2000). fMLP has been shown to induce the activities of ERK1 and ERK2, thus playing a role in neutrophil adherence and respiratory burst activity as well as inducing p38 and contributing to chemotaxis and superoxide anion production (Zu et al. 1998). Recent data obtained in our laboratory (Larbi et al. 2005) indicate that aging is associated with a decrease of ERK and p38 tyrosine phosphorylation in PMN under fMLP stimulation, suggesting a decreased activity of these MAPKs. These alterations could explain the decrease found in effector functions of PMN with aging such as superoxide anion production, as well as chemotaxis. Altogether we assist to an alteration of the human fMLP receptor signalling with aging.

3.2 GM-CSF Receptor

GM-CSF is a powerful modulator of granulopoiesis and the priming of mature PMN to a second stimulation such as fMLP. GM-CSF is able to rescue PMN from apoptosis by interacting with its specific receptor on the PMN plasma membrane. The receptor for GM-CSF is a member of the superfamily of cytokine receptors (Miyajima et al. 1992). Its structure consists of a receptor-specific α subunit and a β subunit (βc) that is shared by the receptors for IL-3 and IL-5 (Miyajima et al. 1993). Although the GM-CSF receptor is not endowed with intrinsic protein kinase activity, its occupation triggers the phosphorylation of its βc subunit on tyrosine residues, most probably by Jak2 (Quelle et al. 1994) and, the phosphorylation of a host of cytoplasmic proteins on tyrosine residues, the expression of early response genes and the proliferation of hematopoietic cells. GM-CSF has been shown to activate three distinct pathways in various cells: 1. the JAK/STAT pathway, 2. the Ras-Raf-1-MEK-MAP kinase pathway and, 3. the PI3-kinase intracellular signalling events (Sato et al. 1993; Watanabe et al. 1997). Recently, the MAPK and PI3K pathways were suggested to be involved with the GM-CSF antiapoptotic effect in PMN (Klein et al. 2000). These signalling pathways modulate the executioner phase of apoptosis, mediated by a family of cysteine proteases, the caspases, as well as members of the bcl-2 family, which are key players in the regulation of apoptosis.

Our recent studies suggest that aging is accompanied by a decrease in GM-CSF-signal transduction (Fortin et al. 2007a). PMN functions were shown to decrease with aging, as well as the antiapoptotic effect of GM-CSF (Fulop et al. 2004). Thus, we also investigated whether the Jak/STAT pathway in PMN under GM-CSF stimulation could be altered with aging. We have demonstrated that activation of the Jak/STAT pathway is altered in PMN of elderly subjects under GM-CSF stimulation. Neither short, nor sustained phosphorylation of Jak2 could be demonstrated and this inability of GM-CSF to induce Jak2 activation was translated in the decreased activation of STAT3 and STAT5. Moreover, the density of GM-CSF receptor β subunit did not change with age. The unchanged βc-subunit expression would assure an equal possibility of signalling in PMN of young and elderly subjects. This is supported by the fact that the physical association between the GM-CSF receptor β subunit and Jak2 did not change neither with aging, nor with GM-CSF stimulation. One other explanation could be an alteration in the membrane composition rendering difficult the mobility of the receptors to facilitate the phosphorylation of Jak2 (Fulop et al. 2004). Recently, the presence of lipid rafts in the cell membrane of PMN was demonstrated (Sitrin et al. 2004; Kindzelskii et al. 2004). These lipid rafts are privileged microdomains in the membrane enriched in cholesterol, sphyngolipids and various proteins, such as signalling proteins (Simons and Ikonen 1997). We found with aging a significant alteration in the composition and properties of lipid rafts of T-cells (Larbi et al. 2004a). In PMN, our group showed in a recent paper an over activation of the protein tyrosine phosphatase SHP-1, a negative regulator of signal transduction, in the lipid rafts with aging. This over activation caused the defects in the activation of the Src Kinase Lyn and contributed to the impaired functions of PMN with aging (Fortin et al. 2006). Moreover, an over activation or a deregu-

lated termination of Jak2 negative regulators, including SOCS, with aging cannot be ruled out and is currently under investigation by, among others, the group of Tortorella (2007). They demonstrated that both SOCS1 and SOCS3 levels were significantly higher in unstimulated neutrophils from elderly individuals than in their younger counterparts and, unlike the neutrophils of young subjects, they did not further increase following GM-CSF stimulation. As a result, a more effective SOCS1 and SOCS3 binding to either the GM-CSF receptor or Jak2, which would largely account for the GM-CSF dependent defect of PI3-K/Akt/ERK activation, might occur in senescent neutrophils. This finding is in line with recent demonstration of elevated SOCS3 levels in resting lymphocytes from elderly donors. Therefore, the increase in this class of inhibitory molecules may be considered as a general phenomenon associated with aging (Tortorella et al. 2007).

We also investigated whether this alteration in the activation of the Jak/STAT signalling pathway could be linked to the decreased antiapoptotic effect of GM-CSF in PMN of elderly subjects. We found that GM-CSF was unable to modulate the Caspase-3 activity in the elderly subjects (Fortin et al. 2007a). Moreover, our results show that AG490 could not modulate the already decreased anti-apoptotic effect of GM-CSF. It is difficult to determine what the exact contribution of the Jak/STAT pathway is, but these results indicate that it plays an important role in the GM-CSF failure to rescue PMN of elderly subjects from apoptosis. Thus, PMN of elderly subjects seem to be in a dominant negative status leading to a decreased response to GM-CSF. This also precludes that if the Jak2 activation is decreased, other downstream signalling pathways could be also altered, such as the PI3-kinase pathway (our unpublished results and Tortorella et al. 2007). Thus, Jak2 might play an upstream and essential role in the signalling cascade to provide survival signal to STATs and other signalling pathways.

PI3-K and the downstream serine/threonine kinase Akt/protein kinase B (Akt/PKB) have a central role in modulating neutrophil respiratory burst activation, chemotaxis and apoptosis. Tortorella et al. (2007) studied the functional activity of the neutrophil PI3-K/Akt pathway in the elderly and found, similarly to the ERK1/2, higher baseline levels of phosphorylated Akt forms and lower GM-CSF-induced phosphorylation of Akt with respect to younger subjects. The link once more between these signalling alterations and the age-related inability of GM-CSF to prolong neutrophil survival emerged from observations using various pharmacological inhibitors such as PD98059, LY294002 or wortmannin. These alterations in the PI3-K/Akt pathways could explain the alterations in the MAPK ERK1/2 activation, as they seem to be activated in succession. In fact, others and we have showed significant alterations in the GM-CSF induced ERK1 and ERK2 tyrosine phosphorylation and, even a higher decrease in ERK1/2 activation with respect to baseline in PMN from elderly subjects compared to young subjects (Larbi et al. 2005). The p38 MAPK pathway was also found altered in PMN from elderly under GM-CSF activation (Larbi et al. 2005).

It is of note that bypassing the GM-CSF receptor by direct inhibition of Caspase-3 was able to rescue PMN from apoptosis in both groups of age (Fortin et al. 2007a). This further indicates that the GM-CSF inability to rescue PMN of elderly

from apoptosis is linked, in part, to the alteration of the signalling pathway that leads from GM-CSFR to Caspase-3. However, it is not the sole factor as there is a significant fraction of residual procaspase-3 in the PMN of the elderly donors after 18h of culture. Moreover, after 18h of culture with GM-CSF there is even a larger fraction of inactive procaspase-3 in the elderly. These results would be surprising if the inhibition of Jak2 by AG490 did resulted in a complete cleavage of procaspase-3 in the elderly as it did in the young donors. We can only hypothesize that the inability of GM-CSF to rescue PMN of elderly from apoptosis is not mediated by the cleavage of procaspase-3 but rather by other mechanisms, especially as the Caspase-3 enzymatic activity has been found to be higher in this paper (Fortin et al. 2007a). Others mechanisms include an altered ratio of antiapoptotic vs. proapoptotic members of the Bcl-2 protein family, such as Bax, BclXL, Bad and A1 (Fulop et al. 2002; Fulop et al. 2004) and, deregulation of the activity of negative regulators of GM-CSF signal transduction like SHP-1 (Fortin et al. 2006). Supporting this notion, GM-CSF has been shown to up-regulate the expression of the antiapoptotic Mcl-1 (Moulding et al. 1998) while interferon-α/γ had similar surviving effects by increasing the expression of the cIAP2 protein (Sakamoto et al. 2005)**.** Moreover, the failure of GM-CSF to sustain STAT3 phosphorylation in the elderly may promote PMN apoptosis by not counteracting the proapoptotic effects of activated STAT1, as it is the case for Mel80 cells. Moreover, these observations in elderly subjects bear a resemblance to the phenomenon observed in T-cells. The T-cell receptor (TCR) signalling is altered leading to deficient proliferation with aging, while bypassing the TCR by PMA and Ca2+ ionophore stimulation restore their proliferative capacity. This could be of importance when we consider the increase of infections with aging and the modulation of PMN function might go through a nonspecific manner.

The Jak/STAT, PI3-K/Akt and MAPK pathways were found to be altered with aging in PMN upon GM-CSF receptor stimulation (Fortin et al. 2007a; Larbi et al. 2005; Tortorella et al. 2007). However, there is no decrease in the GM-CSFR number with aging. The primary alteration could be the Jak/STAT pathway as it seems to regulate all the others. Not only the positive signalling events but also the negative signalling events are altered under GM-CSF stimulation in PMN with aging. This leads to an altered functioning of the PMN with aging concerning apoptosis, chemotaxis and free radical productions.

3.3 Toll-like Receptors

Toll-like receptors (TLRs) are pattern recognition receptors that recognize conserved molecular patterns on microbes and link innate and adaptive immune systems. There exists actually of 10 different TLRs. Ligands for the TLR2 are gram-positive bacteria, while gram-negative bacterial product, LPS, is a ligand for TLR4 and, both of them are found on neutrophils (Remer et al. 2003; Kurt-Jones et al. 2002). The signalling pathways activated by TLRs are broadly classified into MyD88-depend-

ent and independent pathways (Takeda and Akira 2005) as MyD88 is the universal adapter protein recruited by all TLRs, except TLR3. The MyD88-independent pathway of TLR4 signalling is not used in human PMN (Tamassia et al. 2007). The major pathways activated by TLR engagement are using IκB kinase (IKK), MAPK and phosphatidylinositol 3-kinase (PI3-K)/Akt pathways. These pathways regulate the balance between cell viability and inflammation. There are currently four cytosolic adaptor proteins that are thought to play a crucial role in specificity of individual TLR-mediated signalling pathways. Amongst them, TLR4 signalling involves all four adapter proteins, MyD88 (myeloid differentiation primary response gene 88), MyD88 adapter like [MAL; also known as TIRAP (TIR domain-containing adapter protein)], TIR domain-containing adapter protein inducing IFN-β [TRIF; also known as TICAM1 (TIR domain-containing adapter molecule 1)], and TRIF-related adapter molecule [TRAM; also known as TICAM2 (TIR domain-containing adapter molecule 2)] (McGettrick and O'Neill, 2004). The differential recruitment of these adapter proteins by different TLRs form the basis for the specificity in the signalling process activated by them. However, the signal transduction pathway initiated by these interactions is mediated initially by an adaptor molecule, MyD88, recruiting various serine-threonine kinases, IRAKs and finally leading to NF-κB translocation (Kobayashi and Flavell 2004). Among IRAK family proteins IRAK-4 and IRAK-1 play major roles in signal transduction under LPS stimulation. This interaction ultimately results in the secretion of pro-inflammatory cytokines (Cloutier et al. 2007) that recruit the cells of adaptive immune response. That is why the function of TLRs is very important not only for an adequate innate, but also for the adaptive immune response. Moreover, there exists a synergy between TLR2 and GM-CSF receptors (Hayashi et al. 2003).

There exists almost no data concerning the TLRs receptor number and signal transduction in PMN in relation to aging. Renshaw et al. (2002) reported impaired TLR expression and function with aging in mice macrophages. A recent comprehensive evaluation of TLR function in monocytes from older adults was conducted using a multivariable mixed statistical model to account for covariates (van Duin and Shaw 2007). It found that cytokine production after TLR1/2 engagement, which is essential for the recognition of triacylated lipopeptides found in a variety of bacteria, is substantially lower in monocytes from older adults. The up-regulation of costimulatory proteins such as CD80, essential for optimal activation of T-cells, on monocytes from older adults was less for all TLR ligands tested than for cells from young individuals and, the extent of CD80 up-regulation predicted subsequent antibody response to influenza immunization. These and other consequences of aging on human TLR function may impair activation of the immune response and contribute to poorer vaccine responses and greater morbidity and mortality from infectious diseases in older adults. That is why we investigated the TLR4 and TLR2 receptor numbers on PMN of young and elderly subjects by flow cytometry. We found that there is no change in the percentage of PMN expressing TLR4 and TLR2 with aging. Similar results were obtained when we measured, by comparing the Mean Fluorescence Intensity (MFI), the amount of TLR4 or TLR2 receptors expressed by PMN in each age groups (Fulop et al. 2004). These results show that there is no change with aging in the expression of TLR2 and TLR4 receptors on PMN.

One other element, as mentioned above, which recently changed our comprehension of the signalling mechanism through the membrane is the existence of specific signalling microdomains in the membrane, called lipid rafts (Simon and Ilkonen 1997). These were demonstrated in numerous cells and recently in PMN too (Shao et al. 2003). These microdomains, enriched in sphyngolipids, cholesterol and signalling molecules either are parts of, or are recruited to the signalling complexes of the cell membrane. Presently, a few data exist in relation to aging on the existence of these lipid rafts in PMN membranes and how these lipid rafts could be structured and functioning (Fortin et al. 2006, 2007b, 2007c). Therefore, we also studied, whether the unchanged number of TLRs found by FACScan is reinforced by the study of the expression of TLR2 and TLR4 in the PMN membrane lipid rafts. We showed for the first time that LPS not only increases the expression of TLR4 in PMN of young subjects, but increases also its recruitment in the rafts and nonrafts fractions. In contrast, the expression of TLR4 in rafts and nonraft fractions were increased with aging already at the basal status compared to that of PMN of young subjects while no-redistribution occurred after LPS stimulation. It is of note that the apparent increase at basal status of TLR4 expression in the membrane of PMN of elderly could be in accordance with the slight stimulated status of PMN with aging, as already demonstrated (Fulop and Seres 1994). This is also in accordance with the low-grade inflammation present with aging as stated by the inflamm-aging theory of Franceschi et al. (2000). It is of note that no significant changes in TLR2 recruitment occurred in rafts and nonrafts fractions of PMN under LPS stimulation in either young subjects or elderly subjects. These results indicate that even if the number of receptors seems not to change with aging the differential recruitment between raft and nonraft fractions could induce an altered signalling of the receptors, mainly in case of TLR4 under LPS stimulation with aging.

We also studied the early signal transduction events elicited by LPS through the TLR4. The signal transduction of TLR4 under LPS stimulation is mediated at the early phases by MyD88 and IRAKs (Kobayashi and Flavell 2004). We studied the expression of MyD88 and IRAK-1 under LPS stimulation in membrane rafts and nonrafts fractions of PMN. MyD88 was evenly distributed before and after LPS stimulation in the rafts and nonrafts fractions of the membranes in young and elderly subjects. Thus, no differences in the MyD88 distribution could be found with aging, however the quantity of MyD88 in the membrane of PMN of elderly subjects was significantly decreased after stimulation (Fulop et al. 2004). MyD88 is an adaptor protein found very close to the membrane, which could explain that no change in its physical distribution can be observed under stimulation. In contrast, there is a recruitment of IRAK-1 molecules from nonrafts fractions to lipid rafts in PMN of young subjects under LPS stimulation while this recruitment is totally absent in PMN of elderly subjects. It is of note that IRAK-1 was already in the rafts fraction at basal status, in accordance with the slightly activated status of PMN with aging. All these results suggest an alteration in the signal transduction of TLR4 under LPS stimulation with aging either in the redistribution of IRAK-1 signalling protein among rafts and nonrafts fractions, or in the quantity of the MyD88 molecule between rafts and nonrafts fractions. These results provide evidence for a lipid rafts dependant activation of neutrophils via the Toll-like receptor pathway. However,

they cannot explain why the LPS was efficient for chemotaxis of PMN with aging. We can only speculate that either this function is mediated through different signalling pathway(s) or this is a nonreceptor dependent function of LPS, playing the role of a nonspecific chemoattractant.

Altogether, the studies on signal transduction pathways elicited by the stimulation of various PMN receptors suggest that there exists an altered signal transduction in PMN with aging. These alterations does not seem to arise from a change in the receptor number, but most probably from an alteration related to the cell membrane physico-chemical status with aging. We and others have found that the fluidity of PMN with aging, in contrast to other cells, is increasing due to the alteration in the membrane cholesterol/phospholipid composition (Yuli et al. 1982; Alvarez et al. 2001). The cholesterol content does not change while the phospholipid content is increasing. These changes affect the functionality of lipid rafts which are important microdomains for the receptor signalling, as was shown in the case of TLRs and GM-CSF receptors. A dysfunction of the signalling due to age-related changes in actin cytoskeleton function (Rao et al. 1992) has been also suggested to be a contributing factor. Ultimately these changes in signalling decrease the effectors functions of PMN with aging.

3.4 TREM-1

This receptor is a recent addition to the growing members of activating receptors that are members of the Ig superfamily and, is up-regulated at the surface of PMN and macrophages in infection and LPS-induced sepsis in mice (Bouchon et al. 2000). This family mediate their signal transduction with an adapter molecule, for TREM-1 the adapter is DAP12, to elicit a number of common signalling molecules (Bouchon et al. 2000; Klesney-Tait et al. 2006; Tessarz and Cerwenka 2007). Its ligand is still unknown, but the functional responses elicited by the engagement of TREM-1 on monocytes/macrophages and PMN is well known. TREM-1 triggers the release of cytokines and chemokines, ROS production, degranulation and phagocytosis (Bouchon et al. 2000; Bleharski et al. 2003; Radsak et al. 2004; Fortin et al. 2007b). Of note, stimulation of PMN with both TREM-1 and TLR ligands resulted in a synergistic effect on functional responses (Bleharski et al. 2003; Radsak et al. 2004; Fortin et al. 2007b) hence amplifying the inflammatory response and, suggesting potentially aggravating consequences in infections with aging. Inasmuch, our group recently found that TREM-1 and TLR4 colocalized in human PMN upon stimulation with LPS (Fortin et al. 2007b) and, silencing of TREM-1 in macrophages with siRNA resulted in down-regulation of key signalling molecules of the TLR4 pathway (Ornatowska et al. 2007). Thus, emerging data are showing an unsuspected link between the TLR4 and TREM-1 and, it is possible that multimeric complexes are responsible for the recognition of noncytokines mediators of inflammation such as the ligands of TREM-1 (Klesney-Tait and Colonna 2007). We have already evoke the possibilty of an innateosome, which would be responsible for the recognition of LPS and TREM-1 ligands in human PMN, when we showed that stimulation of

either receptors lead to the phosphorylation of IRAK1 and the colocalization at the membrane of TRL4 and TREM-1 (Fortin et al. 2007b). Furthermore, the importance of soluble TREM-1, the shedding of TREM-1 occurs in sepsis or with LPS stimulation of macrophages, in a clinical context it is established that this is a relevant marker for human sepsis and, the use of decoy TREM-1 with blocking ability favored a positive outcome of the resolution of sepsis (Gibot 2006).

So far, only our group studied the impact of aging on the TREM-1-induced functions on cells of the immune system. PMN from elderly donors were found to have impaired response following TREM-1 engagement (Fortin et al. 2007c). Notably, TREM-1 could not prime the production of ROS in the elderly as it did in the young donors and, altered signal transduction of downstream TREM-1-elicited molecules (Akt and PLCγ) was found. Of particular interest, TREM-1 engagement could not reverse PMN survival following incubation with LPS or GM-CSF in the elderly whereas it did in the young. This particular alteration in TREM-1 response could possibly be a contributing factor in the higher incidence of sepsis-related deaths in the elderly population as resolution of inflammation requires clearance of effectors cells. Finally, TREM-1 engagement could not drive the recruitment of TREM-1 in the lipid-rafts of the elderly explaining in part the altered response. Although data exist in human relative to the amount of soluble TREM-1 found in the plasma of patient with or without sepsis, the study was carried only for one age group (mean age 60 ± 15) (Gibot et al. 2004). In keeping with the contributions of TREM-1 in inflammation and the aforementioned alterations in the TEM-1-induced functions in the PMN of the elderly, it would be extremely interesting to have data on lethal outcome of sepsis vs. age of hospitalized patients.

4 Conclusion

PMN are very important part of the immune response towards invading organisms. They are the first line defence being part of the innate immune response and, are essential modulators of the adaptive immune response. It is well known that the incidence of infections is increasing with age. Decrease in specific receptor mediated functions including free radical production, chemotaxis and apoptosis/survival of PMN with aging resulting from an alteration of the positive and negative events in the signalling pathways have been recently demonstrated. These alterations might contribute to the increased incidence of infections with aging. However, these changes in neutrophil functions remain controversial with aging. Elucidation with more rigorous and sophisticated methods of PMN function alterations with aging is needed as these changes could have a great impact on the adaptive immune response. The recent demonstration of lipid rafts in PMN, as being fundamental platforms for signal transduction, will help to better understand the mechanism of age-related signalling changes. These changes should be also taken into account when tentative is made to increase the immune response of the elderly by immunomodulating agents for improving the quality of life of elderly persons.

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Synergistic Effects of Ageing and Stress on Neutrophil Function

Janet M. Lord, Anna C. Phillips and Wiebke Arlt

Contents

Abstract: Although ageing is a complex process, we now know much of what happens with age at the cellular and tissue level. In contrast, our understanding of how the various age-related changes interact to result in frailty and pathology is incomplete. For example, ageing is accompanied by a loss of immune function (Immunesenescence), an increase in the level of circulating proinflammatory cytokines (Inflammaging), a decline in adrenal androgen production (Adrenopause) whilst concurrently peripheral glucocorticoid availability increases. In this article we propose that these changes in combination increase the susceptibility of older adults to the adverse effects of physical and emotional stress, exacerbating the age-related decline in immune competence and exposing the older individual to increased risk of infections. We have focused upon the effects of stress and ageing

J. M. Lord (✉)
MRC Centre for Immune Regulation
Division of Immunity and Infection
Birmingham University Medical School
Birmingham B15 2TT, United Kingdom
Tel: +44 121 414 4399
Fax: +44 414 3599
E-mail: J.M.Lord@bham.ac.uk

A. C. Phillips
School of Sport and Exercise Science
University of Birmingham
Birmingham B15 2TT, United Kingdom

W. Arlt
Division of Medical Sciences
University of Birmingham
Birmingham B15 2TT
United Kingdom

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on neutrophil function, an element of the immune system that has received less attention from immunogerontologists, despite the primary role of neutrophils in fighting bacterial infections and the major contribution of such infections to age-related morbidity and mortality. We propose that physical and emotional stressors elicit an exaggerated response in older adults that synergises with the age-related loss of neutrophil function, to compromise antibacterial mechanisms. Moreover, the molecular basis of this effect may lie with the significant changes in tissue concentrations of cortisol and dehydroepiandrosterone in peripheral target cells including the immune compartment.

1 Ageing, Stress and Infection

It is now well established that the efficiency of the immune system declines with age and this is highlighted most obviously at the functional level by the increased risk of morbidity and mortality from infection in older adults [1–3]. The three major causes of death in the UK in those aged over 65 are cardiovascular diseases, cancer and respiratory disease (Fig. 1). Approximately 1 in 6 older adults will die as a result of the latter, the majority succumbing to respiratory infections. Older adults show a three-fold greater incidence of bacterial dysentery than younger subjects, 50% higher mortality from gram-negative bacterial sepsis, and deaths from gastrointestinal infections, pneumonia and influenza are largely confined to patients over 65 years of age [4, 5]. Age-related reactivation of latent infections previously held in check by the immune system is a further indicator of the age-related decline in immune function (immunesenescence). Thus the incidence of tuberculosis is raised in the elderly, indicating reduced functioning of macrophages [6] and reduced T-lymphocyte function is reflected by reactivation of latent Herpes viruses such as Varicella Zoster (shingles) in older age [7]. Older adults are also at increased risk of postsurgical complications such as infections, which include infections at the wound site; but are dominated by bacterial chest and urinary tract infections [8]. Furthermore, delayed wound healing with age has particular relevance in the context of surgery and has underlying contributions from senescent fibroblasts as well as senescent immune

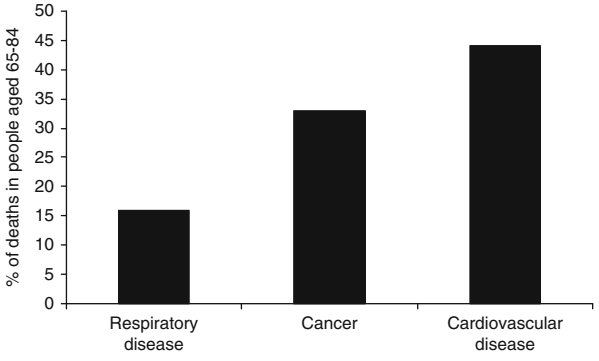


Fig. 1 The figure shows the major causes of death in adults aged 65-84 in the UK, (adapted from data presented in the House of Lords Science and Technology Committee 1st report on Ageing: Scientific Aspects, 2005, Figure 4, page 42)

cells which are key players in the wound healing response (9). The increased risk of infection following surgery may therefore reflect compromised immune and wound healing responses and underline the significant effect of physical stress upon immunity.

It is now well accepted that prolonged exposure to stress, whether psychosocial or physical, has detrimental effects upon immunity. Immune suppression associated with chronic stress has significant clinical consequences, including increased risk of illness and death from infectious disease [10, 11]. In the area of psychosocial stress there is now a solid literature showing reduced immunity, suboptimal responses to infectious agents and vaccines and increased susceptibility to infection in adults following bereavement [12–14] extended care-giving [15, 16], or low social support [10, 17–20]. In an elegant study by Cohen et al. [21] the effect of psychosocial stress on resistance to infection was clearly demonstrated. In this study almost 400 volunteers were exposed to five different respiratory viruses and incidence and severity of infection for each virus was found to be positively correlated with their scores on a psychological stress index. The reader is referred to a recent comprehensive review of this area by Kiecolt-Glaser and Glaser [22].

Moderate physical trauma is also a potent mediator of immune suppression, with 1 in 3 trauma patients succumbing to one or more infections [23]. An extensive retrospective analysis of infections in over 10,000 trauma patients revealed that 32% of patients developed respiratory tract infections and 17% had urinary tract infections [24]. In older adults the most frequent trauma results from falls leading to hip-fractures. Falls are the leading cause of admission into care homes and a frequent cause of death in the elderly population. Again, one of the major health risks associated with falls and hip-fracture is infection, such as osteomyelitis, respiratory infections and infections at the surgical wound site [24–26].

2 Ageing and Neutrophil Function

The high incidence of bacterial infections in older adults is particularly suggestive of a suboptimal neutrophil response, as these leucocytes form the primary response to bacterial, fungal and yeast infections. The preponderance of urinary tract infections in older adults would also support this conclusion. As the effect of ageing on neutrophil function is covered in detail elsewhere in this volume, this topic will be covered only briefly here to allow discussion of the combined effect of age and stress on neutrophil function.

Neutrophils are the dominant leucocyte in the circulation, making up 60% of the white cell count. They are also the shortest lived blood cell, dying by apoptosis approximately 24h after leaving the bone marrow [27, 28]. Their function can be enhanced by proinflammatory cytokines, such as GM-CSF, TNFα and Type 1 interferon, which not only amplify their basic bactericidal functions, such as generation of reactive oxygen species, but also extend their lifespan at sites of infection by inhibiting apoptosis [29, 30]. Neutrophils are recruited to sites of infection

via chemotactic signals, such as the chemokine CXCL8 (also known as IL8). Once in contact with the pathogen they uptake the microbe by phagocytosis mediated via opsonic receptors (CD11b/CD18, CD16, CD32, CD64) that detect complement proteins C3b and C3Bi or antibody coating the microbe. Once inside the neutrophil, pathogens are killed as a result of the generation of reactive oxygen and nitrogen species and the release of a range of proteolytic enzymes from cytoplasmic granules. Phagocytosis and generation of superoxide trigger the death of the neutrophil, which is then removed by macrophages leading to the resolution of inflammation [27].

Comparison of neutrophils from peripheral blood of healthy young and old adults has shown in a majority of studies that chemotaxis is not significantly affected by ageing, with adherence of neutrophils to endothelium [31, 32] and expression of adhesion molecules [31, 33] both unaltered with ageing. In contrast, bactericidal (superoxide generation and degranulation) and phagocytic function is dramatically reduced in neutrophils from older adults [34–37]. For superoxide generation, responses to the bacterial peptide fMLP and to gram-negative bacteria appear to be unaltered by ageing [31, 34, 38], whereas superoxide generation in response to a gram-positive stimulus such as *Staphylococcus aureus,* was significantly reduced in neutrophils from older donors [37]. The latter is of clinical importance bearing in mind the reduced ability of older adults to resolve infections with gram-positive bacteria [39]. The cause of reduced superoxide generation with age is not fully understood, though reduced signaling via calcium in activated neutrophils has been suggested [36] and reduced responsiveness to proinflammatory cytokines such as GM-CSF has also been shown [40, 41]. This is an important finding as cytokines such as GM-CSF prime neutrophil function leading to improved bactericidal responses to bacterial components such as fMLP. In addition, as stated above GM-CSF is also a potent neutrophil survival factor and reduced responsiveness to this cytokine would limit neutrophil lifespan extension at sites of inflammation.

Loss of phagocytic capacity has been investigated reasonably thoroughly. Neutrophils from older subjects retain their ability to phagocytose opsonized bacterial pathogens *per se*, but their phagocytic capacity (phagoctyic index, the number of microbes ingested per cell) is significantly compromised [34, 35, 42–44]. The level of expression of opsonic receptors is known to be a determinant of phagocytic capacity and our data showed a significant reduction in one of the cell surface opsonin receptors (CD16) that binds to antibody coating bacterial pathogens. Taken together these data indicate that neutrophils should be able to respond to chemotactic signals from a site of infection, but will then be severely compromised in their bactericidal function and also their ability to respond to local survival factors such as GM-CSF.

In addition to the obvious consequences of reduced neutrophil function for ability to combat bacterial infections, neutrophils also play a key role in wound healing. In response to tissue injury cytokines are released, including CXCL8, which attract neutrophils to the site. Neutrophils then aid resolution of the damage by removing microbial pathogens and restoring sterility, thus removing the inflammatory stimu-

lus. Whether neutrophils play a positive role in wound healing beyond clearance of pathogens is still a hotly debated topic. Neutrophils release cytokines important for revascularization and tissue repair, such as CXCL8 and VEGF [45], but this has to be balanced with their ability to produce tissue-damaging agents if they persist at a sterile wound site. Reduced phagocytosis of microbes will lead to persistence of inflammation and prevention of wound healing by the presence of high levels of inflammatory cytokines. Although this aspect of innate immunesenescence has received less attention, it is potentially a significant factor in the development and persistence of ulcerated wounds in the elderly.

3 Stress and Neutrophil Function

Stress, whether physical or psychological, is broadly sensed by 2 endocrine regulatory systems, the Hypothalamic-Pituitary-Adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) system. Stress induces the release of catecholamines (adrenalin and noradrenalin) from the adrenal medulla and the sympathetic nervous system and, mediated via an increased pituitary ACTH secretion, results in an acute increase in cortisol and dehydroepiandrosterone (DHEA) release from the adrenal cortex. Catecholamines and cortisol are both immune suppressive [46, 47], whereas DHEA is a precursor to sex hormones and is generally thought to be immune enhancing [48–52], though evidence for the latter is less substantial due to lack of data generated in humans and human cell-based systems. In particular, all DHEA replacement studies in humans have been carried out in healthy older subjects only [53–55] and not under conditions of stress in which this hormone may play its vital role in counteracting the negative effects of cortisol (discussed below).

As the effects of stress on adaptive immune functions are dealt with separately in this volume, the focus here will be upon neutrophil function in response to stress.

3.1 Acute Stress

The impact of acute psychological stress on neutrophils has received little attention within the immune literature. In animals, acute psychological stress can be applied in a variety of ways including inescapable intermittent electric shock, overcrowding, or restraint in an enclosed space. Acute psychological stressors in humans usually involve brief laboratory-based tasks such as public speaking or mental arithmetic in front of an audience and/or under time pressure, or the cold-pressor test (submersion of the hand in ice-cold water), although some studies have used examination stress as a short-term stressor. Overall, the literature suggests that periods of acute stress have beneficial effects on neutrophil function. Mice that had received 2.5 hours of restraint stress showed increased infiltration of neutrophils into a surgically implanted sponge in comparison to unstressed control mice [57]. In addition, an

increase in neutrophil adhesion and aggregation has also been shown to be induced by short periods of inescapable foot shock in rats [58], an acute anticipatory stressor in healthy young adults [59, 60], and stroop and mirror tracing tasks in men aged 30–59 [61].

Acute stress has also been shown to modulate phagocytosis: periods of social conflict stress between mice for less than one day resulted in increased phagocytosis by neutrophils and other phagocytic cells in comparison to nonstressed mice [62]. When neutrophils are activated, they undergo a respiratory burst and produce toxic superoxides that kill the pathogens they have phagocytosed. In humans, a 15 minute time pressured stress task (Raven's Advanced Progressive Matrices) induced an immediate significant increase in the number of activated neutrophils relative to resting baseline and in comparison to a nonstressed control group. This increase in activation state had returned to baseline 10 minutes following the end of the stress task. A comparison of neutrophil function in students between final examination week and nonexamination weeks showed that the short-term stress of examinations was associated with an increase in neutrophil superoxide production [63, 64], and this increase was maintained at 2–3 weeks post examinations [63]. In rats, superoxide production was increased in response to 1 hour of open field stress [65]; and superoxide production at the site of inflammation following experimental *E.coli* injection was observed to be higher in rats previously exposed to inescapable tail shock in restraint tubes for two hours in comparison to non-stressed rats. In the latter study, stressed rats also showed a complete resolution of the inflammatory response to the infection two weeks faster than control nonstressed rats, potentially indicating a stress-induced elevation in neutrophil response resulting in more effective bactericidal activity [66]. This increase in neutrophil function parallels other acute stress induced changes in nonspecific immunity such as the increased production of secretory immunoglobulin A [67].

In summary, acute stress appears to have an overall positive impact upon neutrophil function, particularly when acute stress is applied in the context of an inflammatory challenge, although the mechanisms of such effects are unclear at present.

3.2 Chronic Stress

In contrast to acute stress, the effects of chronic exposure to stress are detrimental to immune function. A meta-analysis of thirty years literature on the effect of stress on the immune system concluded that chronic stress such as bereavement or physical trauma resulted in suppression of cellular and humoral immunity and increased susceptibility to infection [68]. As with acute stress there is very little information relating to neutrophil function and the research emphasis has been placed upon adaptive immune responses. However, chronic stress and depression in cancer patients is associated with neutrophilia, but also with decreased neutrophil phagocytic ability and raised cortisol levels [69]. Intense or long duration exercise is also associated with raised circulating cortisol and adrenalin, together with reduced neutrophil bac-

tericidal responses namely degranulation and superoxide generation [70]. Our own studies of the effect of physical stress (limb or hip-fracture) on neutrophil function confirmed a profound neutrophilia in response to stress, but also a significant suppression of superoxide generation in response to a bacterial peptide challenge which correlated with raised cortisol levels [71].

Glucocortoids are likely to be major mediators of the negative effects of chronic stress upon neutrophil biology and function [72]. For example, studies of the effects of cortisol infusions in humans have shown a profound neutrophilia, which is achieved in part by the inhibition of neutrophil apoptosis thus extending neutrophil lifespan in the circulation [73, 74]. Cortisol can also enhance G-CSF mediated stimulation of granulopoiesis in the bone marrow [75], further contributing to raised neutrophil numbers in response to cortisol. Unfortunately the potential benefit of an increased level of circulating neutrophils with raised cortisol is not realized, as cortisol also inhibits neutrophil chemotaxis and extravasation [76]. The clinical significance of this observation is seen in studies that reported reduced neutrophil chemotaxis in trauma patients and a strong correlation with increased incidence of infection [77, 78]. In vitro studies also suggest that the suppression of neutrophil superoxide generation after trauma is mediated by cortisol. We and others have shown that superoxide generation by cytokine-primed neutrophils in vitro was suppressed by cortisol [71, 79], though we found no effect of cortisol on phagocytic function (S.K. Butcher, unpublished data). Taken together these data indicate that raised cortisol levels will impact negatively upon neutrophil function, which could in turn increase susceptibility to bacterial infections.

The meta-analysis carried out by Segerstrom and Miller [68] also revealed that the loss of immunity in response to stress was much greater in older adults, which in turn concurs with reports of excess infection-related morbidity and mortality in older trauma patients [80–82]. Our own studies have compared responses to stress in young and old trauma patients and revealed that the detrimental effect of physical stress was most marked in older adults [71, 83], supporting an influence of age upon stress mediated suppression of neutrophil responses. That neutrophil function was affected by chronic stress in patients with cancer, an age-related disease, adds further weight to this proposal. Synergy between the effects of stress and immunesenescence on immune function, has also been proposed in relation to psychosocial stress and the immune system [22, 84] and there is now a real need to compare the differential effects of stress on a broad range of immune responses in young and old subjects.

4 Ageing and Stress Hormones

While cortisol secretion by the adrenocortical zona fasciculata appears to remain largely unchanged throughout life [85], adrenal dehydroepiandrosterone (DHEA) secretion from the adrenal zona reticularis exhibits a characteristic, age-associated pattern. Intraindividual maximum levels of DHEA and its sulphate ester DHEAS are

reached during early adulthood, followed by a steady decline throughout adult life, eventually decreasing to 10–20% of previous maximum levels by 70–80 years of age [86, 87]. This age-associated decline in DHEA synthesis has been termed "adrenopause", which is somewhat imprecise given that adrenocortical glucocorticoid and mineralocorticoid secretion is maintained without change across the lifespan. Interestingly, an age-associated secretion pattern of DHEA is only observed in humans and higher nonhuman primates [88, 89] and it is important to recognize that rodent adrenals are not capable of DHEA secretion, yielding only very low circulating DHEA concentrations of primarily gonadal origin, thereby limiting the suitability of rodents for studies on the significance of DHEA for human physiology and disease. Adrenopause is independent of menopause, and it occurs in both sexes; it shows high interindividual variability and has been suggested to be associated with a macroscopically visible decrease in size of the adrenal zona reticularis [90]. There is also a suggestion of an age related increase in senescent and apoptotic cells within the zona reticularis, though whether this contributes to loss of cells in this region of the adrenal cortex, or might influence functional activity of the zona reticularis cells, has not been established [91].

DHEA secretion exhibits a diurnal rhythm similar to that of cortisol and ongoing age has been shown to be associated with an attenuation of the diurnal rhythm and the pulse amplitude of DHEA secretion [92]. Furthermore, the adrenal stress response seems to be partially impaired with ageing, with a significant reduction of acute DHEA release following an acute exogenous ACTH challenge whilst the cortisol response remains intact [93]. In young healthy subjects it has been shown that an acute psychosocial stressor such as an arithmetic challenge or public speaking test results in an acute rise in cortisol [94], but neither the impact of this on DHEA release nor its modification in the aged has been investigated to date. We have recently shown that acute sepsis leads to an up-regulation of both cortisol and DHEA [95], however, comprehensive data on chronic exposure to physical or psychological stress are lacking.

Whilst cortisol mediates its action via the cytosolic glucocorticoid receptor that, once activated, translocates to the nucleus and initiates the transcription of glucocorticoid effector genes, the exact mechanisms underlying the actions of DHEA and its sulphate ester DHEAS still remain controversial. High affinity binding sites for DHEA have been described in murine and human T-lymphocytes [96, 97] and human vascular endothelial cells [98, 99], but their specificity as opposed to active androgens is still debated. DHEA has also been shown to have neurosteroidal properties and exerts stimulatory effects on NMDA receptors and inhibitory effects on $GABA_A$ receptors in the brain [87]. However, the current view is that the majority of its actions are mediated indirectly, via downstream conversion to sex steroids and other steroids of potentially distinct activity including the putatively immune modulatory steroids androstenediol, androstenetriol and 7α-OH-DHEA [100] (Fig. 2).

DHEA represents a paradigm for prereceptor metabolism as its action will mainly depend on the expression of enzymes responsible for its conversion to other steroids in the specific target cell of interest. Lymphocytes and macrophages have been shown previously to express steroidogenic enzymes involved in the downstream

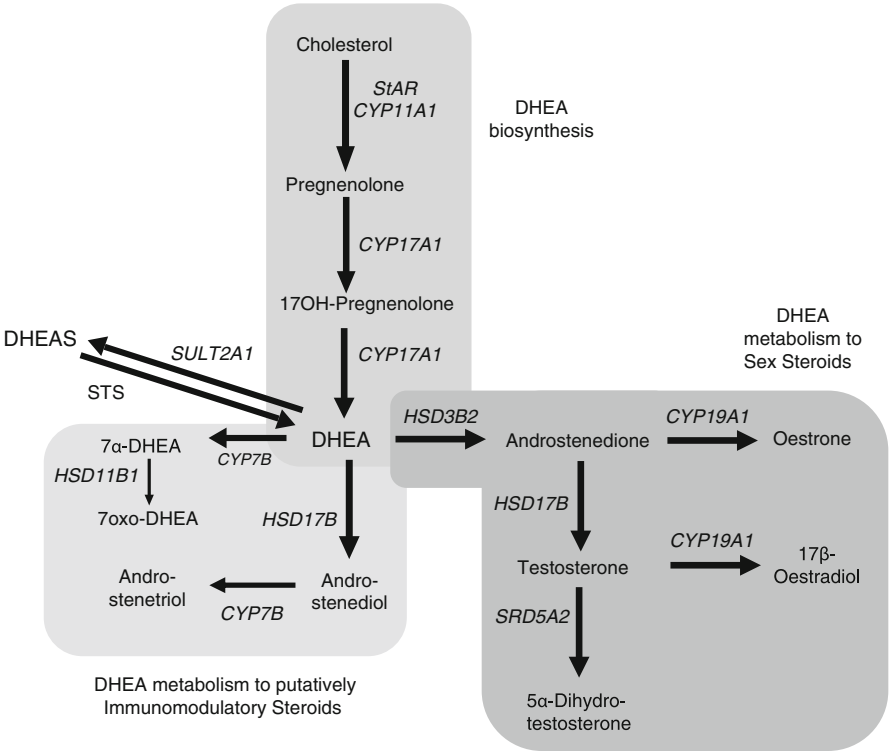


Fig. 2 Dehydroepiandrosterone (DHEA) biosynthesis from cholesterol via StAR (steroidogenic acute regulatory protein), CYP11A1 (side chain-cleavage enzyme) and CYP17A1 (17α-hydroxylase/17, 20 lyase) as well as its downstream metabolism to sex steroids and potentially immunemodulatory steroids via HSD3B (3β-hydroxysteroid dehydrogenase Type 1 and 2), HSD17B (17β-hydroxysteroid dehydrogenases), SRD5A1 (5a-reductase Type 1 and 2), CYP19A1 (Aromatase), CYP7B (7α-hydroxylase) and HSD11B1 (11β-hydroxysteroid dehydrogenase Type 1). Lipophilic DHEA can be converted to its hydrophilic sulphate ester DHEAS by SULT2A1 (DHEA sulphotransferase) and back by STS (steroid sulfatase)

metabolism of DHEA [101–103], but their presence in neutrophils has not been determined. We have recently shown that the expression level of these enzymes changes with ageing and demonstrated enhanced conversion of DHEA to androstenediol as well as increased androgen activation by 5α-reductase in lymphocytes from older men as compared to young men [104]. Concurrently circulating levels of DHEAS and testosterone were significantly lower in the older men, suggesting that the up-regulation of steroidogenic enzymes in the lymphocyte compartment may be a counter-regulatory event aiming to maintain intracellular availability of androstenediol and dihydrotestosterone.

Although circulating cortisol levels do not change significantly with aging, intracellular availability of active glucocorticoids within the peripheral target cells including immune cells may well be altered with age. The major regulatory switch controlling tissue-specific activation of glucocorticoids is the enzyme

11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1), which converts inactive cortisone to active cortisol [105] (Fig. 3). 11β-HSD1 has two activities; in vivo it mainly acts as an oxoreductase, activating cortisone to cortisol, whereas in vitro it mostly exhibits dehydrogenase activity, converting cortisol to inactive cortisone. 11β-HSD1 is anchored in the endoplasmic reticulum (ER) membrane and has its catalytic domain directed towards the lumen of the ER. Only recently it has been elucidated that its oxoreductase activity is dependent on NADPH generation by the endoplasmic reticulum luminal enzyme hexose-6-phosphate dehydrogenase (H6PDH) [106, 107] (Fig. 3). It is well established that expression and functional activity of 11β-HSD1 can be up-regulated by inflammatory cytokines, e.g., in adipose tissue and bone [108, 109]. As ageing leads to a cytokine profile that is more proinflammatory, with raised levels of IL-1β, IL-6 and TNFα [110], it can be readily hypothesized that increased activity of 11β-HSD1 would lead to increased tissue-specific glucocorticoid availability and action. A precedent for age related changes in 11β-HSD1 expression may relate to the brain, where an age related increase in 11β-HSD1 might explain why cerebrospinal fluid cortisol concentrations rise with age despite unchanged serum cortisol levels [111]. This concept has been convincingly supported by recent human in vivo data demonstrating an improvement in cognitive function by inhibition of 11β-HSD1 activity [112].

Interestingly, it has been shown that 11β-HSD1 is expressed in immune cells and that 11β-HSD1 expression is induced in monocytes upon differentiation to macrophages [113]. By contrast, intracellular glucocorticoid activation by 11β-

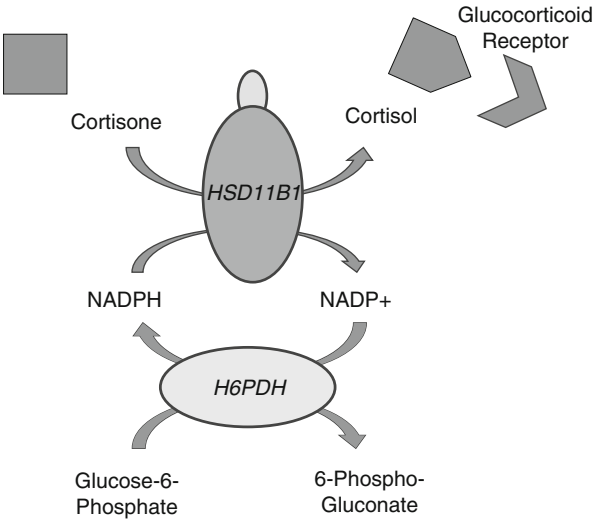


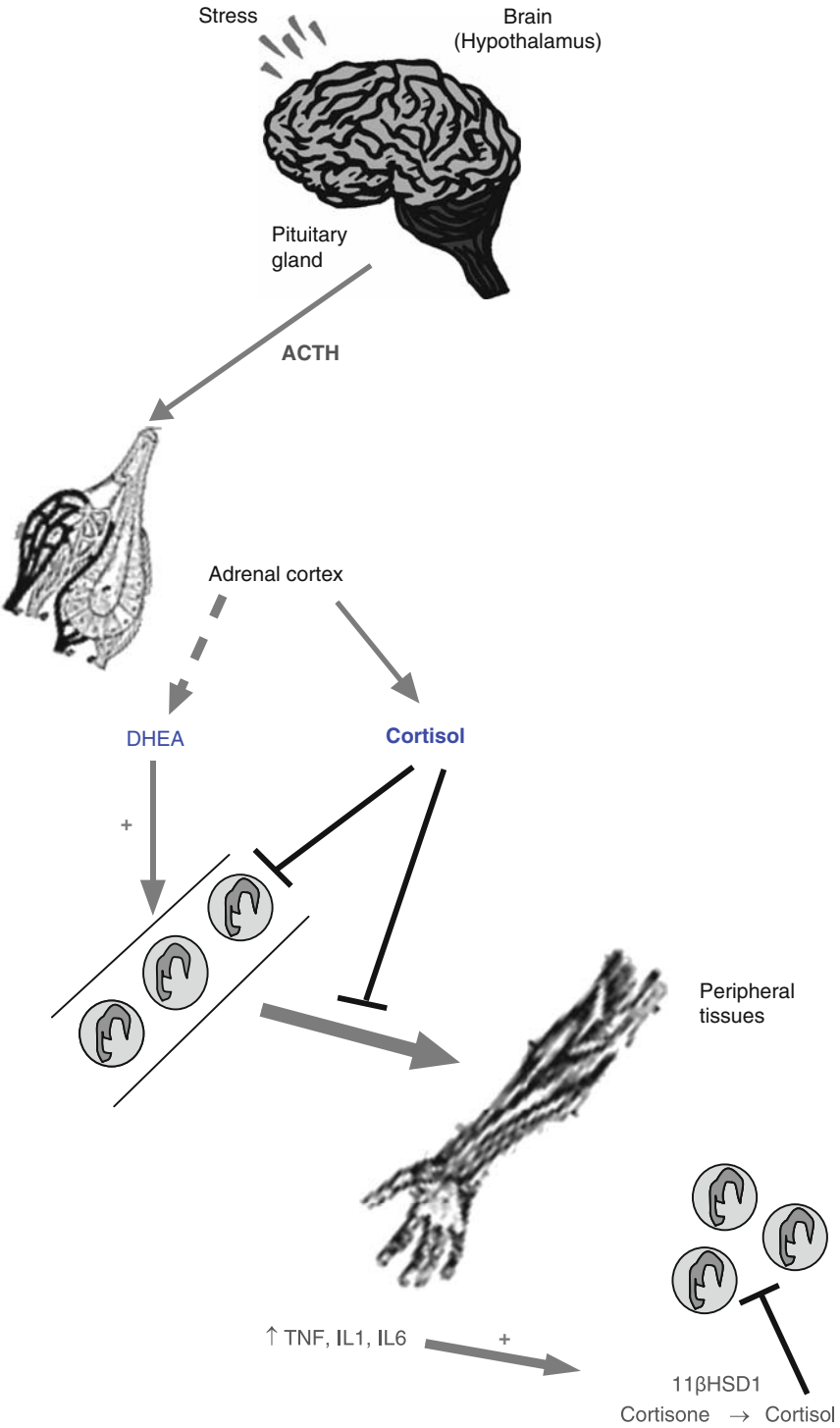
Fig. 3 The oxoreductase activity of 11β-hydroxysteroid dehydrogenase (HSD11B1) converts inactive cortisone to active cortisol that binds and activates the glucocorticoid receptor (GR). HSD11B1 is predominantly an oxoreductase *in vivo*, however *in vitro*, following disruption of the endoplasmic reticulum integrity, mainly acts as a dehydrogenase, inactivating cortisol to cortisone. HSD11B1 is anchored in the ER membrane with its catalytic domain towards the ER lumen and its oxoreductase activity is dependent on NADPH delivery by the intraluminal enzyme hexose-6-phosphate dehydrogenase (H6PD)

HSD1 oxoreductase activity sharply declines during the maturation of monocyte-derived dendritic cells [114]. Whether macrophage and dendritic cell function are differentially modulated by cortisol remains an interesting possibility. The loss of DHEA with age, accompanied by increased 11β-HSD1 in immune cells and tissues induced by inflammaging, may thus result in increased glucocorticoid activity in inflamed tissues, further dampening the immune response in older adults. In addition, inhibition of 11β-HSD1 decreases cortisol half-life [115] and the observed increase in cortisol half-life in the elderly (by as much as 40%) may be a reflection of enhanced 11β-HSD1 activity [116]. Thus we postulate that ageing represents a state of tissue specific cortisol excess in the context of normal circulating levels and that this will impair the peripheral immune response in tissues throughout the body.

5 Stress Hormones and Neutrophil Function

The vast majority of literature on this topic is focused upon the immune suppressive activity of cortisol, with little attention paid to DHEA or its downstream metabolites. Moreover, very few studies of stress hormone effects on immune cells have considered neutrophils. The active glucocorticoid cortisol exhibits a variety of immune suppressing effects [117], which appear to be counteracted by DHEA. For example, it has been suggested that DHEA and glucocorticoids have opposing effects on T-helper cell 1 (Th1)/Th2 balance [118] with evidence for protection of a Th1 cytokine profile by DHEA [119]. In rodents, DHEA antagonizes dexamethasone-induced suppression of lymphocyte proliferation and prevents glucocorticoid-induced thymic and splenic atrophy [120]. DHEA and dexamethasone may have opposing effects on dendritic cell differentiation [121]. DHEA and the glucocorticoid receptor antagonist RU486 equally reverse the suppressive effects of glucocorticoids on immune function [49]. In vitro studies utilizing human immune cells have demonstrated an increase in IL-2 secretion [52] and natural killer cell cytotoxicity [122] following exposure to DHEA. Conversely, DHEA has been shown to inhibit IL-6 release and circulating DHEAS levels have been shown to negatively correlate with serum IL-6 [123, 124]. Thus DHEA appears to counteract the changes in cytokine secretion characteristically observed with ageing, i.e. decreased IL-2 and increased IL-6 levels. DHEA replacement in patients with adrenal insufficiency and thus pronounced DHEA deficiency has been shown to increase the number of circulating regulatory T-cells [125], but this study did not provide details on functional activity.

In relation to neutrophil function, as described above cortisol is a potent suppressor of neutrophil bactericidal responses inhibiting neutrophil superoxide generation [79]. Our own work has confirmed these reports and shown that DHEAS was able to enhance neutrophil superoxide generation in vitro and to overcome the suppressive effects of cortisol on primed neutrophil superoxide generation [71]. Indirect support for the ability of DHEA to counteract the immune suppressive effects of cortisol in vivo, comes from animal studies of DHEA supplementation. For example, dietary


Stress
Brain
(Hypothalamus)
Pituitary
gland
ACTH
Adrenal cortex
DHEA
Cortisol
+
Peripheral
tissues
↑ TNF, IL1, IL6
+
11βHSD1
Cortisone → Cortisol

DHEA supplementation of rodents exposed to physical trauma resulted in reduced mortality from the trauma induced sepsis [126]. The latter may be due not only to improved neutrophil function in the presence of DHEA, but also to moderation of the shock response which can produce excessive neutrophil accumulation in tissues leading to nonspecific tissue damage. In this respect DHEA has been reported to down-regulate induction of adhesion molecule expression by LPS [127]. It is thus clear that both cortisol and DHEA appear to modulate neutrophil function, but much more research is necessary to determine the extent of these effects and their mechanisms, i.e. whether the effects of DHEA are direct or via downstream metabolites.

Peripheral actions of glucocorticoids may also be modified by DHEA. DHEA reverses glucocorticoid-associated immune changes after trauma/haemorrhage in mice, concurrently leading to normalisation of elevated corticosterone levels [128], which may suggest an effect upon the prereceptor modulation of glucocorticoids, specifically on 11β-HSD1. Down-regulation of 11β-HSD1 expression and activity by DHEA was recently demonstrated in rat hepatocytes [129], in murine adipocytes [130] and in human skeletal muscle [131]. Thus in the situation of ageing we predict an increase in glucocorticoid action in immune cells due to enhanced tissue-specific activation by 11β-HSD1 whilst the potentially counteracting DHEA pool diminishes due to the age-associated decline in adrenal DHEA production.

In summary, there is still a paucity of data generated in human based systems that informs about DHEA-induced immune effects, in particular there are few studies investigating the effect of DHEA or DHEAS on neutrophil function. The data available to date do however suggest that DHEA can counteract the immune suppressive effects of corticosteroids, including suppression of neutrophil function [132].

6 Ageing, Stress and Neutrophil Function

We propose that the age-related immune and endocrine changes outlined above have specific implications for resilience to stress in older adults. We hypothesize that the combination of adrenopause, leading to a relative preponderance of cortisol over DHEA, with increased tissue levels of 11β-HSD1 resulting in raised peripheral cortisol availability and an already reduced immune defence against infection, leave this population particularly susceptible to the negative effects of stress on immunity. For example, relative to age-matched controls, older adults exposed to the chronic stress of being the primary caregiver for a partner with dementia have

Fig. 4 Stress is sensed by the hypothalamus which secretes corticotrophin releasing hormone, stimulating the pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH acts upon the adrenal gland causing release of glucocorticoids (cortisol) and DHEA into the circulation. Cortisol suppresses neutrophil function, including extravasation, whereas DHEA counteracts the effects of cortisol and promotes neutrophil function. With age the ability to produce DHEA is reduced (indicated by the dashed line) giving a relative excess of cortisol. Raised levels of inflammatory cytokines induce 11β-HSD1 expression in peripheral tissues increasing conversion of inactive cortisone to active cortisol. The overall effect of age and stress is to diminish the antibacterial actions of neutrophils, thus increasing susceptibly to these infections in older adults

shown a variety of immunological decrements. These include a reduced response to pneumococcal pneumonia vaccination and poorer *in vitro* NK-cell cytotoxicity [133, 134]. More recently, *in vivo* assessments of immune function, such as healing rates of experimentally administered wounds and antibody response to vaccination, have been used to provide clinically relevant outcome measures of the effects of stress on immunity. These studies have supported the previous work suggesting that psychosocial stress is associated with reduced immune functioning in older populations. For example, older adults providing long-term care-giving have shown delayed wound healing in the mouth [135]. Further, experimentally induced punch biopsy wounds took significantly longer to heal in chronically stressed older caregivers and immune cells from the caregivers produced significantly less of the cytokine IL-1β in response to stimulation *in vitro* than the cells of women who were not caregivers [135]. Although an interaction between stress and ageing has not, to our knowledge, been tested directly in the context of psychological stress, there is evidence that younger care-givers of multiple sclerosis patients do not demonstrate reduced antibody responses to vaccination compared to controls [136]. In addition, influenza vaccination responses were reduced in chronically stressed older adults to a much greater extent than young adults [137].

Physical bodily trauma, such as hip-fracture, can also be considered as a chronic stressor, and is associated with decrements in immune function. For example, the experience of hip-fracture in adults aged over 65 years was associated with diminished neutrophil function (generation of superoxide) and a significant incidence (43%) of bacterial infection [83]. Interestingly, this effect of trauma on neutrophil function and infection rates was not observed in younger adults with limb fractures, suggesting that this immune impact of stress is worsened by the presence of immunesenescence. This finding supports the hypothesis that nonacute stress, whether physical [132] or psychosocial [84] exacerbates the negative influence of ageing on the immune system.

7 Conclusions and Future Directions

Ageing has been defined classically as the increasing frailty of an organism with time that reduces its ability to deal with stress, resulting in increased probability of disease and death. The age-related loss of innate immune function, including reduced neutrophil bactericidal function, contributes to such frailty by increasing susceptibility of older adults to bacterial, fungal and yeast infections. Chronic stress is also detrimental to immune function and although the literature regarding neutrophils is sparse, the negative effects of cortisol are well documented. We propose that the age-related increase in proinflammatory cytokines will increase tissue levels of cortisol via induction of 11β-HSD1, contributing to peripheral immune suppression in old age. Furthermore, the negative effects of cortisol appear to be modified by the counter stress hormone DHEA and we suggest that the loss of this

hormone with age mediates a synergistic down regulation of innate immune function by chronic stress and immunesenescence.

There is currently a paucity of experimental data concerning differential responses to stress with age and in particular the effects of stress on neutrophil function in older adults. Future intervention strategies to improve neutrophil function at times of stress may therefore usefully target the altered HPA axis, either by elevating circulating DHEA levels or by functional antagonism of cortisol at the cellular level.

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Antigen Presenting Cells

Role of Dendritic Cells in Aging

Anshu Agrawal, Sudhanshu Agrawal and Sudhir Gupta

Contents

Abstract: Immunesenescence is characterized by a decline in immune functions which is responsible for majority of morbidity and mortality associated with aging. The possible consequences of this progressive aging of the immune system are an increase in autoimmune phenomena, incidence of malignancies and predisposition to infections. Innate immune system is the primary defense against invading pathogens. Moreover, it also initiates and modulates the functions of adaptive immune system. This review focuses on the age-associated changes in the functions of dendritic cells, the major antigen presenting cells of the innate immune system.

1 Introduction

The immune system is composed of two major defenses—1) innate immune or nonspecific defense mechanisms consisting of cells such as granulocytes, macrophages and dendritic cells (DCs) and proteins such as cytokines which allows an extremely rapid response to pathogens. 2) The adaptive immune or specific immune defense mechanisms consist mainly of cells such as T- and B-lymphocytes and follow the innate immune response. This response is exquisitely tailored and specific

S. Gupta (✉)
Medical Sciences I, C-240, University of California, Irvine
CA 92697, USA
Tel: (949) 824-5818
Fax: (949) 824-4362w
E-mail: sgupta@uci.edu

A. Agrawal · S. Agrawal
Division of Basic and Clinical Immunology, University of California
Irvine, CA 92697

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to the particular pathogen and is responsible for generating long-term memory against subsequent challenges. These two defense mechanisms are interlinked together and the nature of innate immune response dictates the nature or quality of adaptive immune response. The proper function of both systems is thus essential for generating effective immunity. Adaptive immune functions are known to be severely compromised with increasing age and believed to be the majo r cause for immunosenescence [18, 23, 26, 27, 35, 36, 42, 44, 60]. The knowledge regarding the contribution of innate immune cells such as DCs in immunosenescence is still in its infancy. This review summarizes the findings on DC functions in aging.

2 Dendritic Cells

Dendritic cells (DCs) are the most potent of antigen-presenting cells [6, 58] of the immune system present at various portals of entry of pathogens like skin, airways etc., sensing pathogens, ready to initiate and amplify the immune response. DCs serve as critical mediators of both immunity and tolerance [6, 57, 58] by virtue of their ability to ascertain that inflammatory immune responses against commensals of the physiologic skin microflora, ingested food antigens, or inhaled airborne microorganisms are prevented while potent immune responses against harmful pathogens are sustained.

DCs can be activated by various stimuli including microbes, dying cells, and inflammatory cytokines. An array of Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and intracytoplasmic NOD-like receptors (NLRs) in DCs aid them in sensing pathogens [5, 28, 50]. The sensing and capture of antigen by DCs initiates their differentiation and maturation. During maturation they lose their antigen capturing capacity and upregulate the expression of MHC and costimulatory molecules, thus becoming the efficient antigen presenting cells [6, 57, 58]. Maturation of DCs also results in the upregulation of CCR-7 which allows them migrate to T-cell areas in the lymphoid organs where antigens are presented to the T-cells, initiating an adaptive immune response. In addition to an up-regulation of various markers, activation of DCs initiates an inflammatory response through secretion of a broad array of cytokines and other inflammatory mediators which allows them to communicate between themselves and other cells of the immune system, exerting a broad influence on the immune system [6, 50]. For example, DCs can dictate the type of T-cell responses by virtue of the cytokines they secrete in response to a stimulus. IL-12 or IL-23 secretion by DCs [1, 13, 34] primes TH1/TH17 responses while IL-10 gives rise to TH2 or T-regulatory type of responses [2, 3].

Maintenance of tolerance is another key function of DCs [57, 58]. Under steady state conditions DCs continuously sample self-antigens from dying cells. However, no immune response is initiated since these do not activate DCs. These immature DCs interact with T-cells in the absence of costimulation leading to T-cell anergy or the development of T-regulatory cells. The presence of danger signals such as proinflammatory cytokines can cause DCs to mature resulting in break of tolerance. The proinflammatory cytokines and other inflammatory mediators that are increased

during aging can modulate functions of dendritic cells affecting the magnitude and quality of both innate and adaptive immune responses [10, 19, 30, 45].

3 Dendritic Cell Subsets

In humans, two major subsets of DCs [6] have been identified that function differently in both the innate and adaptive immune responses: myeloid DCs (mDCs), – interstitial DCs and Langerhans cells are found in peripheral tissue, secondary lymphoid organs and blood, and plasmacytoid DCs (pDCs) are present in the blood and secondary lymphoid organs. mDCs are professional APCs with a strong capacity to prime naive T-cells and to induce and regulate T-cell responses through secretion of IL-12. pDCs on the other hand are characterized by their plasma cell-like morphology, low phagocytic capacity and production of large amounts of Type I interferons in response to viral, bacterial and parasitic infections [38]. The two subsets of DCs differ in their expression of highly conserved microbial pattern recognition receptors (PRRs), known as Toll-like receptors (TLR). Circulating pDCs express TLR1, 6, 7, 9 and 10, but not TLR4 [29, 51], while blood mDCs express TLR1, 2, 3, 4, 5, 6, 7, 8 and 10, but not TLR9 [51].

4 DCs in Aging

Aging is associated with multiple changes in the cytokine microenvironment that could have either inhibitory or stimulatory effect on the activation and/or maturation of DCs [9, 10, 19, 30, 45, 49]. The increased TNF-α and prostaglandins would result in premature DC activation altering their antigen uptake capacity [30]. Similarly elevated IL-10 and glucocorticoid levels in aging may result in the suppression of activation of DCs [10, 45]. A change in the microenvironment such as age-associated increase in proinflammatory cytokines like TNF-α, may act as a trigger for maturation of DCs, and in combination with apoptotic cells may lead to immune activation and associated inflammation.

Studies on DCs in aging in humans have been focused primarily on the function of myeloid DC subset because it was believed to be the major DC subset involved in T-cell priming.

5 DC Numbers and Phenotype

Most studies in humans have reported no change in numbers or phenotype of DCs in aging [39, 56, 61]. In our study we found normal numbers and phenotype of circulating and monocyte-derived DCs (MDDCs) in aged humans [4]. However,

Della-bella et al. [15] reported progressive decline in the number of circulating mDCs with age while there was no change in pDC numbers. They also found that DCs from aged individuals have a more mature phenotype with an increased proportions of cells expressing CD86 and CD83 as compared to young individuals. Recent mouse studies by Grolleau–Julius et al. [22] and Tesar et al. [59] also did not report any–significant difference in the numbers and phenotype of blood or lymphoid DCs. However, HLA-DR expression on peripheral blood DCs and DCs from a strain of senescence-accelerated mouse (SAMP-1) was found to be reduced [24]. A few earlier studies in mice document a decrease in the number Langerhan cells in the skin [11, 14, 55].

6 DC Pathogen Sensing and Cytokine Secretion

H. Influenzae and *Streptococcus pneumoniae* infections account for substantial mortality [31, 41] in aged individuals resulting in increased susceptibility to infections with age. Alterations in PRR functions in aging may impair activation of the immune response and contribute to poorer vaccine responses and greater morbidity and mortality from infectious diseases. Proinflammatory cytokines are increased during aging. A greater understanding of PRR functions in aging is extremely relevant in view of the interest in TLR agonists as therapeutic agents not only for infections, but also for allergic, autoimmune, and malignant diseases.

Amongst the PRRs, TLRs are most studied in aging because of their important role in clearing viral and bacterial pathogens. Numerous studies have investigated the expression of these receptors on the innate immune cells of both humans and mice. Alterations in TLR expression pattern in aging seem to be cell specific with monocytes showing decreased expression of certain TLRs while dendritic cells appear to express similar level of TLRs as the young.

Some earlier studies [8, 48, 54, 61, 62] had reported decreased expression and function of TLRs in macrophages from aged mice; however, recent studies by Tesar et al. [59] found that TLR expression and function (in vivo) is intact in myeloid DCs and macrophages from aged mice. They found that both circulating mDCs and bone-marrow derived DCs from aged and young mice expressed comparable levels of maturation markers following activation with various TLR ligands. The levels of cytokine secretion between the groups were also comparable. In contrast, Grolleau-Julius et al. [22] reported decreased secretion of TNF-α and IL-6 and increased IL-10 secretion from bone-marrow derived DCs from aged mice. The reason for this discrepancy is presently unclear. Previous studies [39, 56] have reported comparable levels of activation and cytokine secretion by MDDCs from the aged and young subjects following TLR stimulation. Our studies [4] found no differences in TLR expression in MDDCs from aged and young subjects at the gene level (Affymatrix analyses) and protein level (TLR4). There was no difference in the expression of CD40, CD80, CD83, CD86 and HLA-DR on MDDCs before and after activation with TLR-4 ligand, LPS between

aged and young subjects. However, we observed an increased secretion of TNF-α and IL-6 from MDDCs from aged compared to young subjects, when stimulated with TLR-4 or TLR8 ligand. The levels of IL-10, IL-12p40 and IL-12p70 were comparable between the two groups. Della bella et al. [15] report decreased IL-12 secretion from LPS-stimulated circulating mDCs in humans. Unpublished data from our laboratory indicates that circulating mDCs in the aged subjects also secrete higher levels of TNF-α and IL-6 upon TLR stimulation. In summary, TLR expression and function in DCs during aging appears to be intact except an increased secretion of proinflammatory cytokines TNF-α and IL-6. High IL-6 and TNF-α levels are poor prognostic factors for a number of age-associated diseases. For example, higher IL-6 levels lead to the production of C-reactive protein which is identified as a major risk factor for myocardial infarction. Increased serum IL-6 and C-reactive protein play a major role in Type-2 diabetes; rheumatoid arthritis and osteoporosis, all diseases which show increased incidence with age [12, 16, 43]. Likewise there is positive correlation between IL-6 levels and congestive heart failure. Increase in another proinflammatory cytokine TNF-α has been found to be associated with higher incidence of arteriosclerosis in older men [47]. Thus overactivation of the dendritic cells in response to TLR may be contributing to age-associated inflammation.

7 Antigen Capture

Phagocytosis is the major mechanism used by DCs to remove pathogens and cell debris and therefore is important for maintaining both immunity and tolerance [57, 58]. Our studies [4] have shown that both, the phagocytosis of dextran beads and pinocytosis of Lucifer Yellow dye was found to be impaired in MDDCs from aged subjects when compared to young. This suggested that MDDCs from aged displayed reduced capacity to capture antigen via both receptor-dependent and independent mechanisms. In addition to foreign antigens, DCs also capture and present self-antigens in the periphery [25, 57, 58]. In fact the uptake of apoptotic cells by DCs in the periphery and presentation to T-cells in the absence of costimulation, is considered to be the major mechanism of maintenance of peripheral self-tolerance. Our investigations indicated that DCs from aged individuals were also impaired in their capacity to phagocytose apoptotic cells [4]. Impaired clearance of apoptotic cells in aging would lead to accumulation of apoptotic cells which will become necrotic and lyse to release auto-antigens such as nucleic acids, heat shock proteins, HMGB1, ATP and uric acid along with other cell debris. In contrast to apoptotic cells which are known to inhibit maturation of DCs, necrotic cells lead to activation of DCs and secretion of proinflammatory cytokines. Thus these auto-antigens can be taken up by DCs and presented to T-cells leading to inflammation and autoimmunity associated with age. Therefore, a reduction in the phagocytic capacity of DCs with age would not only result in reduced clearance of infections but would contribute to age-associated loss of peripheral self-tolerance, autoimmunity and chronic inflammation.

8 Migration of DCs

Following activation, DCs migrate to T- and B-cell areas after activation in order to induce effective cellular immune responses. Stimulation of immature DCs with TLR ligands results in the down-regulation of CCR6 and up-regulation of CCR7, which enhances their ability to migrate from the peripheral tissues to the draining lymph node. CCL21 and CCL19 both bind to the CCR7 receptor and are potent chemoattractants for mature DCs [32]. Mice deficient in CCR7 are unable to mount effective T-cell immunity [20]. Relatively few studies have addressed the question of migration of DCs in aging. We determined the migration of DCs from aged and young human subjects using a transwell system where the DCs in the upper chamber migrate through a transwell of defined pore size to the lower chamber in response to a chemokine gradient [4]. We observed that DCs from aged subjects were impaired in their capacity to migrate in response to CCL19 and SDF-1 compared to DCs from young. This was not a consequence of reduced CCR7 or CXCR4 expression. Neither did we observe a significant difference in the secretion of basal level of the chemokines from DCs between aged and young individuals. Bhushan et al. [7] observed significantly impaired migration of LCs in response to TNF-α in elderly subjects. The same group also found decreased TNF-α induced LC migration in aged animals. Choi and Sauder [11] reported decreased LCs mobilization and the subsequent accumulation of DCs in the regional lymph nodes in aged mice in response to topical challenge with a chemical agent; however, contact hypersensitivity responses were not compromised. Linton et al. [37] have reported in vivo impaired migration of DCs from aged mice to the draining lymph nodes, in a TCR transgenic mice model. They suggest it to be due to both intrinsic defect of DCs and aged microenvironment. Contrary to above studies, Pietschmann et al. [46] observed normal trans-endothelial migration of peripheral blood myeloid-enriched lymphocyte-depleted cells in elderly subjects. This could be due to the difference in the model system. Impaired migration of DCs has also been observed in mice [14, 17]. Since the migration of DCs to lymph nodes is pivotal to the establishment of the immune response, reduced migration may contribute to age-associated immune dysfunction.

9 Dendritic Cells and Adaptive Immunity

DCs play a key role in sensing and processing microbial information and directing the differentiation of naïve lymphocytes to effector cells suitable against particular types of infection. They have the unique capacity to prime naïve T-cells among the antigen presenting cells of the body, therefore they are critical for mounting immune responses against new antigens. The engagement of TLRs on DCs leads to an increased expression of MHC–peptide complexes and costim-

ulatory molecules, as well as the production of immunomodulatory cytokines, all of which have a profound effect on T-cell priming and differentiation. The up-regulation of costimulatory molecules on DCs dictates the decision between tolerance and immunity. Antigen presentation by DCs in the absence of costimulation results in the generation of anergic and/ or regulatory T-cells [25]. DCs also dictate the nature of TH (TH1, TH2, Treg, TH17) response generated through the type of cytokine secreted by them. For example, IL-12 from DCs induces T-cells to secrete IFN-γ [2] while IL-23 will induce IL-17 from T-cells [13, 34]. Secretion of IL-10 by DCs on the other hand induces either a TH2 or a T-regulatory type of response [2, 3]. It is thus clear that any alteration in the function of DCs with age, would affect T-cell responses.

There is some controversy regarding capacity of DCs in old age to stimulate T-cells. Earlier studies in aged mice demonstrated decrease in antigen presentation and T-cell priming capacity of DCs in the lymph nodes [17, 33, 55]. However, the two recent studies in mice are contradictory. Study by Tesar et al. [59] suggests the intrinsic defect in T-cells during age is responsible for the age-associated reduced T-cell function. Grollaeu-julius [22] on the other hand found old bone-marrow derived DCs less effective than young DCs in stimulating syngeneic ova–specific CD4 T-cell proliferation. They also reported a decrease in tumor regression in mice treated with the ovalbumin peptide-pulsed aged DCs than with ovalbumin peptide-pulsed young DCs.

Similar to the findings of Tesar et al. in mice [59], MDDCs from elderly subjects were not impaired in their capacity to induce T-cell responses. Steger et al. [56] and Grewe et al. [21] reported that DCs from young and aged subjects have similar stimulatory capacity to induce proliferation of T-cell lines developed in long-term cultures. Our preliminary studies with MDDCs show reduced proliferation of young T-cells when cultured with aged DCs. The reason for this discrepancy is not clear.

10 Plasmacytoid DCs and Aging

Studies described above are all focused on the mDC subset from either the blood or in vitro monocyte-derived DCs. Except for a report by Schodell et al. [53] documenting a decrease in the number and IFN-α secretion by pDCs in aging, virtually nothing is known about the functions of the pDC subset in aging. pDCs are key players in the elimination of infections and upon activation produce extremely high amounts of Type I IFNs. Type I IFN production by pDCs regulates the cytotoxic potential of NK and CD8 T-cells [38]. It also induces differentiation of B-cells to plasma cells. The NK and CD8 cytotoxicity are reduced with age along with a reduction in specific antibody responses. Studies of pDC functions with age may help in identification of mechanisms responsible for the reduced B and T-cell functions and the associated increased susceptibility to infections.

11 Conclusion

Though the numbers and phenotype of DCs are relatively unchanged during aging dendritic cell functions are altered with age resulting in an enhanced secretion of proinflammatory cytokines. This increased in proinflammatory cytokines may be due decreased sensitivity of the cells to these cytokines, a phenomenon similar to what is observed with insulin resistance. Reduced phagocytosis and migration on the other hand increases the susceptibility of the elderly to infections. Therefore, it appears that dysfunction of DCs may contribute to T-and B-cell immunosenescence and chronic inflammation associated with aging.

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Phenotypic and Functional Changes of Circulating Monocytes in Elderly

Lia Ginaldi and Massimo De Martinis

Contents

Abstract: Immunosenescence has been envisaged as a situation in which the specific immune system deteriorates with age, while the innate immunity is negligibly affected and, in some cases, almost upregulated. Ageing represents a state of paradox where chronic inflammation is associated with declining immune responses. This peculiar finding, known as inflammageing, is mainly sustained by cells of the innate immunity. One of the key constituents of the innate immune system are monocytes. Therefore, although the age-related changes in the specific immunity are commonly considered the hallmarks of immunosenescence, the central role of the complex remodelling of first line defence cells, such as monocytes, is gradually emerging. For example, chemotaxis and phagocytosis, as well as antigen processing and presentation, are depressed, whereas cell activation and the secretion of inflammatory cytokines, such as IL-1, IL-6, TNF, are markedly increased. Changes in the expression of functionally important cellular receptors on monocyte surface can also contribute to the modification of immune function characteristic of the elderly.

1 Monocyte Biology and Function

One of the key constituents of the immune system are monocyte–macrophages. Monocytes originate in the bone marrow and migrate through blood to body tissues as macrophages. Monocytes therefore represent the immature macrophages when in transit from bone marrow to tissues. These cells, also known as mononuclear phagocytes, share a common precursor with neutrophils. Through the expression

Prof. L. Ginaldi (✉) · M. De Martinis
Department of Internal Medicine and Public Health
University of L'Aquila
Coppito-67100 L'Aquila, Italy
Tel.: +39 0861 211395
Fax: +39 0861 211395
E-mail: liaginaldi@alice.it

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of a series of transcription factors differentiation takes place [67]. Human bone marrow produces approximately 5×10^9 monocytes per day. Under the influence of cytokines, a small number of macrophages in tissues differentiate and, depending on the anatomical sites, may become osteoclasts (bone), Kupffer cells (liver), microglia (brain), etc., all of which exhibit unusual morphological features and functional capacities. Monocytes proliferate in the presence of growth factors, such as monocyte colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-3. When these cells are needed at the inflammatory loci, in order to become activated and fully functional, they must interact with interferon-γ (IFN-γ), a cytokine released by activated T-lymphocytes that interacts with the specific receptor [52, 71].

B- and T lymphocytes have specific recognition systems (immunoglobulins and the T-cell receptor, respectively), that interact with specific antigens. This mechanism allows the survival of just the small number of lymphocytes that are needed to recognize and remove foreign material. However, monocytes are members of innate immunity and thus present nonspecific systems on their cell surface to recognize and discriminate self from nonself.

Monocyte–macrophages play a crucial role in immune response and act through several mechanisms: (a) directly, by destroying bacteria, parasites, viruses and tumor cells; (b) indirectly, by releasing mediators, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), etc., which can actìvate other cells; (c) by processing antigens and presenting digested peptides to T lymphocytes; and (d) by repairing tissue damage [70].

Monocyte–macrophages can produce an impressive array of cytokines, chemokines, enzymes, arachidonic acid metabolites, and reactive radicals upon activation. Many of these functions appear to antagonize or counter each other. These cells can clearly enhance or suppress adaptive immune responses. Macrophages display both proinflammatory and antiinflammatory functions, produce metalloproteinases and inhibitors of these metalloproteinases, and produce toxic radicals that contribute to tissue cell destruction as well as cytokines that promote tissue regeneration and wound healing. All of these functions are not expressed simultaneously but are thought to be regulated such that macrophages display a balanced, harmonious pattern of functions [79].

In the classic acute inflammatory response, blood monocytes enter the damaged tissue shortly after neutrophils. Encounter with bacteria, their products, and damaged tissue results in the activation of pro-inflammatory activities, such as the production of TNF-α, IL-1, and IL-6 and the secretion of metalloproteinases [102].

In addition to the inflammatory, clearance and tissue regenerative activities, macrophages also play a critical liaison role in the communication between the innate and adaptive immune systems. Macrophages can display antigen presenting activity and phenotype [46, 95] and the inflammatory milieu created by monocyte–macrophages can significantly impact the maturation of myeloid dendritic cells and thus influence the nature of the adaptive immune response that will be elicited [47, 117]. A function-polarizing synergy can develop between T-cells and macrophages wherein the functional pattern displayed by the macrophages influences the nature

of the adaptive immune response and the nature of the adaptive immune response (TH l vs. TH2) influences the functional pattern displayed by the macrophages. Th l cytokines, such as IFN-γ and TNF-α, promote inflammatory and cytotoxic activities of macrophages. In contrast, Th2 cytokines, such as IL-4 and IL-10, promote anti-inflammatory and/or tissue regenerative activities. Ligation of surface receptors such as CD40, TNF-αR, or Toll-like receptors (TLR) on macrophages initiates signal cascades that provide a strong activating stimulus for macrophage function. IFN-γ selectively upregulates LPS-induced inflammatory cytokine production and NOS and oxidase expression while down-regulating other functions, such as arginase and PGE2 and LTC4 production [75, 103, 104].

2 The Impact of Ageing on Monocyte Function

The immune system is affected by ageing, causing an increased susceptibility to infections and mortality, as well as a major incidence of immune diseases and cancer in the elderly. Because mononuclear phagocytes are an essential component of both innate and adaptive immunity, altered function of these cells with aging may play a key role in immunosenescence [98, 50].

Human immunosenescence has been envisaged as a situation in which the specific immune system deteriorates with age, while the innate immunity is negligibly affected and, in some cases, almost upregulated [46]. Aging represents a state of paradox where chronic inflammation is associated with declining immune responses [1, 107].

Inflammageing is considered the common and most important driving force of age-related pathologies, such as neurodegeneration, atherosclerosis, diabetes and sarcopenia, among others, all of which share an inflammatory pathogenesis [36]. The cell types more involved in the inflammatory processes and therefore in the inflammageing are cells of the innate immunity, such as monocytes/macrophages. For example, adhesion of monocytes to the arterial wall, via specific cell surface adhesion molecules, is an important early event in the development of atherosclerotic lesions [112]. Similarly, the increased incidence of tumours in the elderly has been related to a modified antitumour innate defence [63]. In addition, the interface between innate and adaptive immunity, implicates that many of the changes of monocytes influence the initiation of specific immune responses. Impaired ability of antigen presenting cells (APCs) to stimulate T-cells in elderly has been shown [13, 29]. Therefore, although the age-related changes in the specific immunity are commonly considered the hallmarks of immunosenescence, the central role of the complex remodeling of first line defence cells, such as monocytes, is gradually emerging. Some functions of the innate immunity are depressed in the elderly, while many other functions are upregulated, exerting a global and peculiar reshaping. For example, while chemotaxis and phagocytosis, as well as antigen processing and presentation, are depressed, cell activation and the secretion of inflammatory cytokines, such as IL-1, IL-6, TNF, or mononuclear phagocytic cell specific enzymes, are markedly increased [24, 43, 46, 66].

Healthy elderly subjects and centenarians show a decreased susceptibility of monocytes to oxidative stress-induced apoptosis [77]. The respiratory burst of monocytes during ageing decreases between 45% and 70%. Scavenger receptor activity and the expression of apolipoprotein E are reduced in healthy elderly men [37] as is the inflammatory wound healing response, which may be related to poor expression of cell adhesion molecule-1 [6].

Reports on the impact of advanced age on the recruitment of monocytes into excisional wound sites vary from observations of no significant effect to observatians of long delays in attainment of peak monocyte numbers [25].

Chemotactic activity decreases with advanced age [82, 106]. Phagocytosis and clearance of infectious organisms is also reduced with advanced age [2, 4, 12, 73]. Expression of class II MHC and antigen presentation by macrophages have been reported to be reduced in aged rodents and humans [39, 69, 118]. The production of fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), epithelial growth factor (EGF), and transforming growth factor-beta (TGF-β) are reduced and/or delayed, as is the expression of their corresponding receptors [136]. The result is a delay and/or deficiency in reepithelialization, collagen deposition, and angiogenesis in excisional wounds of the elderly.

Haematopoietic stem cells age and have a limited functional lifespan [40]. This may explain the hypocellularìty observed in the bone marrow of elderly people [81]. Of particular interest is the decrease with age of CD68 positive cells, which are markers of macrophage population [70].

Cell lifespan may be regulated by multiple factors. Recently, telomeres and telomerase have been implicated in the regulation of replicative lifespan [56]. Several studies using peripheral blood mononuclear cells consisting of 10–15% monocytes and 60–70% lymphocytes have shown that these structures shorten with age at a rate comparable with that of purified lymphocytes. Mature monocytes do not undergo further cell division after activation. Thus, the variations in telomere length in monocytes with aging may reflect the changes in telomere length in hematopoietic progenitor cells. While mature monocytes do not express telomerase, myeloid progenitor cells do [111].

There are several potential molecular mechanisms that may affect monocyte ageing. An important and universal mechanism that leads to a wide spectrum of intracellular damage during aging are the reactive oxygen species (ROS), which are natural by-products of cellular metabolism. Exposure to ROS may lead to structural changes in macromolecules that impair their function, such as cross-linking of intracellular and intramitochondrial structural and functional proteins, carbohydrates and the oxidation of fats and lipids in membranes. A likely cause of monocyte ageing is the acquisition of defects in genomic DNA. This may occur through a reduced ability to repair even small amounts of DNA damage or very stringent requirements on DNA repair machinery for the maintenance of DNA fidelity, or both. In addition, an increased number of spontaneous mutations may occur, thereby producing DNA damage [105].

3 The Interface Between Innate and Adaptive Immunity

The complex process of immune activation is dependent on the close participation of T-cells and APCs. APCs are responsible for uptake, processing, and presentation of antigen to T-cells. Impaired ability of APCs to stimulate T-cells in elderly has been shown. Expression of costimulatory molecules that assist in the efficiency of cell to cell communication may be altered in old subjects and thus alter cytokine production by APCs, which regulates downstream T-cell effector functions [46, 48, 86]. However, some studies have shown enhanced antigen presentation by APCs from healthy elderly, associated with increased levels of IL-10 and IL-12. It is hypothesized that this upregulation in IL-12 production by APCs may be compensatory to an inherent age-related decline in T-cell function to maintain immunocompetence [46].

Antigenic presentation is a very complex phenomenon involving the formation of the immunological synapse via the activation of the T-cell receptor (TCR) and coreceptors. This interaction determines whether the interacting T-cell becomes tolerant or proliferates and differentiates into a functional effector T-cell. The capacity for immune synapse formation between APC and T-lymphocyte is altered with age. This may be partly due to an alteration in the membrane properties and costimulatory molecules of the cells of the innate immune system with ageing. The innate immune system also influences the adaptive immune response through the timing, type and strength of cytokines produced. Ageing is associated even in healthy persons with a non specific increase in the production of proinflammatory cytokines originating from monocyte to macrophages [50].

Dendritic cells (DCs) are the major APCs responsible for initiating an immune response. Agrawal et al. [1] compared the innate immune functions of monocyte-derived myeloid DCs from elderly subjects with DCs from young individuals. They showed that, although phenotypically comparable, DCs from the aging are functionally different from DCs from the young. In contrast to DCs from the young, DCs from elderly individuals display (1) significantly reduced capacity to phagocytose antigens via macropinocytosis and endocytosis as determined by flow cytometry (2) impaired capacity to migrate in vitro in response to the chemokines MIP-3β and stromal cell-derived factor-1 and (3) significantly increased LPS and ssRNA-induced secretion of TNF-α and IL-6, as determined by ELISA. Investigations of intracellular signalling revealed reduced phosphorylation of AKT in DCs from the ageing, indirectly suggesting decreased activation of the PI3K pathway. Because the PI3K-signaling pathway plays a positive regulatory role in phagocytosis and migration, and also functions as a negative regulator of (TLR) signaling by inducing activation of p38MAPK, this may explain the aberrant innate immune functioning of DCs from elderly subjects. Results from real-time PCR and protein expression by flow cytometry demonstrated an increased expression of phosphatase and tensin homolog, a negative regulator of the PI3K-signaling pathway, in DCs from the aging. Increased phosphatase and tensin homolog may thus be responsible for the

defect in AKT phosphorylation and, therefore, the altered innate immune response of DCs from elderly humans [57, 58, 83, 116].

Della Bella et al. [21] analyzed the number, phenotype and function of peripheral blood DCs from elderly subjects by using flow cytometric methods that allow cell characterization directly in whole blood samples. They demonstrated that the number of myeloid DCs progressively declines with age. This finding was accompanied by a decrease of CD34+ precursors and increase of circulating monocytes, suggesting that the entire differentiation process of APCs is partially dysregulated in the elderly. DCs from aged individuals also appeares to have a more mature phenotype and impaired ability to produce IL-12 upon stimulation [85].

The frequency of CD34+ cells progressively declines with age, suggesting that in aged subjects a reduced availability of these cells may contribute to reduce the frequency of DCs [99]. On the other hand the frequency of monocytes, that not only may differentiate into DCs but also represent another main population of professional APCs, shows a progressive increase with ageing. Therefore, the entire differentiation process of APCs is partially dysregulated in the elderly. The analysis of the plasmatic levels of factors known to affect the differentiation of monocytes and DCs from their precursors demonstrates in the elderly increased levels of TGF-β, which does promote the maturation and differentiation of monocytic cells [33]; and increased levels of VEGF, which does impair the differentiation of CD34+ cells into mature DCs [63]. The percentage of peripheral blood dendritic cells (PBDCs) expressing the costimulatory molecule CD86 and the maturation marker CD83 are slightly increased in the aged individuals. The easier explanation for this finding is that this partial activation and maturation of PBDCs may be sustained by the increased inflammatory activity that accompanies ageing [65]. The finding of higher plasma levels of TNF-α in the aged subjects seems to corroborate this hypothesis.

4 Age-Related Phenotypic Changes of Monocytes

The phenotype of monocytes in the elderly is consistently remodelled [43]. Changes in the expression of functionally important cellular receptors can contribute to the modification of immune function characteristic of the elderly [42, 45].

Our previous studies [23, 24, 44] demonstrated important cell adhesion receptor modifications on lymphocyte subsets in the elderly, related to peculiar lymphocyte dysfunctions. A significant expansion of CD14dim CD16bright subpopulation of circulating monocytes in elderly subjects, that may indicate a state of in vivo activation, has been demonstrated [96]. Cell adhesion molecules (CAMs) are surface receptors mediating cell-cell and cell-matrix interactions [102]. CAMs are essential molecules involved in chemotaxis, phagocytosis and killing of microbes and neoplastic cells. The increased susceptibility of elderly people to cancer and infections could be partly explained as a failure in such basic immune defence functions [24].

Since leukocyte adhesion molecules play important roles in mediating a wide variety of leukocyte functions, age-related changes in their expression on monocyte

surface could be partially responsible for immune dysfunctions during senescence. Chiricolo et al. [17] documented a decrease in monocyte subpopulations bearing the adhesion molecule CD11a/CD18 and an increase in CD44 antigen density on monocytes in the elderly. These changes might be an event in the mechanism leading to the decreased lymphocyte proliferative response in vitro and to other immunological dysfunctions reported in old subjects.

Considering the central role of the innate immunity in the process of immunosenescence and the involvement of CAMs in the great majority of leukocyte functions, we studied the expression of CD50 and CD62L adhesion molecules in peripheral blood monocytes in the elderly. Such adhesion receptors mediate important cellular functions. CD50 (ICAM-3) is an Ig-related molecule which functions both in cell adhesion and activation processes [20]. Moreover, ICAM-3 is important in the initial scanning of the APC surface by T-cells and, therefore, in generating the specific immune response [78]. CD62L (L-selectin) is an important leucocyte homing receptor which is required to initiate leukocyte capture, rolling and adhesive interactions. In response to inflammatory stimulation, the endothelium expresses a distinct ligand for L-selectin that is sufficient for capture of leukocytes. CD62L is up regulated on circulating leucocytes early after injury and L-selectin mediated signalling may directly initiate or amplify neutrophil activation and localization selectively at sites of inflammation. The percentages of monocytes expressing CD62L is decreased in the elderly, whereas its density expression is unchanged. CD50 expression on monocytes from old subjects show a peculiar attitude: its density expression decreases whereas the number of positive cells is expanded. CD50 is associated with tyrosine kinase activity and functions as a ligand for LFA-1 [24].

CD50 on the surface of APCs plays an important role in the initiation of the immune. Its lower expression on monocytes could therefore contribute to the impaired antigen presentation in the elderly. On the other hand, the increased number of CD50 positive monocytes in the elderly, despite its decreased density expression at a per cell level, could be interpreted as a tentative to counteract the inability to mount strong immune responses [76].

Under some conditions, engagement of surface adhesion molecules induces activation of intracellular signaling cascades (outside-in signaling) that causes altered cellular function and responses. CD50 stimulation on monocytes potently induces secretion and spreading of chemokines (MIP-1alpha, IL-8, and MCP-1 by monocytes and IL-8 by neutrophils) [62]. The increased production of chemokines in the elderly is a well known phenomenon [89]. Therefore CD50 downregulation, as the consequence of its engagement by specific ligand and consequent activation, is the first cellular step in chemokine production.

CD50 and CD62L are released to the medium upon cell stimulation. The increased proportion of granulocytes and monocytes lacking CD62L and the downregulation of CD50 intensity expression may suggest a state of in vivo activation. The presence of soluble CAMs in plasma might serve as a physiological adhesion regulatory system to prevent undesirable leukocyte cell–cell interaction or the attachment of leucocytes to endothelium [22]. Serum levels of solubile cell adhesion receptors are increased in patients with several pathologic states, as well as in the elderly. There-

fore CD50 and CD62L shedding from the cell surface of activated monocytes could be interpreted as a tentative to counteract the dangerous effects of an excessive chronic inflammation in the elderly. However, the increased proportion of CD62L negative monocytes in the elderly leads to an impairment in cell adhesion which is the first line of response to acute inflammatory stimuli. This phenomenon likely contributes to the increased susceptibility to acute infections of elderly people.

5 Hormone Modulation and the Stress Response in Senescent Monocytes

Several hormones, differentially modulated during ageing, can regulate immune cell function. For example, ageing is associated with various degrees of insulin resistance together with reduced immune cell activity. Since monocytes express insulin receptors, the perturbation of insulin pathway has been proposed as possible pathogenetic mechanism in the immune derangement in the elderly. Walrand et al. [110] measured circulating monocyte receptor expression and density using flow cytometric detection. The density of monocyte insulin receptors was not affected by age. Therefore, notwithstanding the presence of insulin receptors on monocytes, insulin dysfunction pathway has a limited action on monocyte function during ageing.

Alterations in retinoid metabolism and thyroid dysfunction occur with senescence. Vitamin A and retinoid acid have a wide variety of profound effects on growth, epithelial tissue differentiation and homeostasis, and are involved in maintaining an efficient immune system [74]. An age-related hypo-activation of the retinoid and thyroid nuclear pathways has also been demonstrated on monocytes and lymphocytes [31].

Monocytes play early roles in triggering an acute inflammatory response to many stressful conditions. The expression of leucocyte L-selectin increases during acute stress events such as injury and is temporally related to an early neuroendocrine response. Adrenaline up-regulates whereas TNF-α down-regulates the surface expression of L-selectin on monocytes [90]. The stress response in the elderly is impaired as well as the secretion of stress response hormons (cortisol, catecolamines) thus contributing to the decreased CD62L expression on both monocytes and granulocytes with consequent inhability to trigger acute inflammatory reactions. The downregulation of CD62L on the cell surface could also be the consequence of the increase of proinflammatory cytokines, such as TNF-α, which characterizes immunosenescence [24].

The effect of age in the production of heat shock proteins (Hsp) is very controversial. Hsp are highly conserved proteins and their synthesis is ubiquitous. Constitutive and stress-inducible Hsp play diverse roles in cellular function. Under normal physiological conditions constitutively synthesised Hsp act as molecular chaperones modulating protein folding, assembly, intracellular localisation, secretion, and degradation. When cells endure stress such as high temperature, exercise, oxidative stress, osmotic stress, and inflammation, the expression of inducible

Hsp is increased and these proteins participate in protein refolding and protection, in dissolving aggregated proteins, and in targeting them for degradation. Hsp27 is able to induce an increase in cellular glutathione levels, which works together with ascorbic acid and coenzyme Q as a redox buffer for cellular protection. With ageing there is a general decline in the capacity of cells to respond to stressors and oxidative insult [80].

Some investigators have reported an increase in the basal levels of Hsp with age, which is indicative of an adaptive response to cumulative intracellular stress during ageing and may be associated with increased oxidative stress. On the other hand, a decrease or no effect of age on Hsp basal levels have also been reported [30].

Njemini R et al. [80] investigated the effect of age and inflammation on the induction of Hsp27 in human peripheral blood monocytes, using flow cytometry. There is an age-related decrease in the level of Hsp27, which disappeares in the presence of inflammation. A relationship between the circulating levels of C reactive protein (CRP), IL-6 and TNF-α with Hsp27 levels exists, indicating that cytokines are able to influence the production of Hsp27. The basal level of Hsp27, measured as mean fluorescence intensity (MFI) or as percentage of Hsp27 producing cells, is inversely related to age, for both lymphocytes and monocytes.The expression of Hsp27 as well as Hsp70 is high in monocytes compared to other leukocyte subsets. Because Hsp27 is up-regulated following oxidative stress a likely explanation for this phenomenon is the higher capacity of monocytes to induce ROS and thus to promote oxidative stress. Since Hsp27 production increases with inflammation, it is possible that it exerts some antiinflammatory or immune modulatory effects on leukocytes. Inflammation results in the neutralisation of the age induced Hsp27 repression. Acute phase factors, which mediate the regulation of Hsp genes by interacting with several signaling pathways are most likely involved in this process. TNF-α might be one such factor, since there is a correlation with the percentages of monocytes producing Hsp27. This observation is compatible with the known proinflammatory tendency that is observed during ageing, and might explain the lower values for Hsp27 in the elderly compared to the younger subjects.

6 Monocyte–Macrophage Subset and Cytokine Dysregulation

Monocyte–macrophage heterogeneity has been recognized recently, and an imbalance in subsets could be a reason for the difference between the young adult versus the aged. Mononuclear phagocytes have been subdivided into M-1 and M-2 phenotypes depending on their ability to produce NO and proinfíammatory cytokines (M-1 type) or antiinflammatory agents such as IL-1RA and arginase (M-2 type), suggesting a possibility that one of these types of macrophages accumulates in the spleens of the aged [41, 75]. NOS-2 and arginase, respectively, unique to M-1 and M-2 macrophages, are reduced in macrophages from the aged. It has also been shown that macrophages can be activated by IL-4, leading to suppression of proinflammatory cytokines and enhanced expression of major histocompatibility

complex class II (MHC II) genes as well as IL-1RA [47]. As the aged have been shown to have an increased incidence of TH2 T-cells [55], it is conceivable that the macrophages in the aged have markers of IL-4 activation. Mosser and colleagues [3] identified a uniquely hyporesponsive macrophages in spleens from the aged, which has profound influences on immune responses to polysaccharide antigens and may affect the overall ability of the aged to generate an inflammatory response necessary to contain infections. Several studies have examined the capacity of phagocytic mononuclear cells to produce cytokines or chemokines [4, 79]. There are reports of increases, decreases or no effects of age on cytokine release by monocytes, either spontaneously or after LPS stimulation [80, 81]. The decreased response of monocytes from aged persons to LPS in relation to the production of IL-6 and TNF-α has been associáted with deficiencies in the activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and deficient expression of c-Fos and c-Jun [57, 59, 91].

Ageing is associated with progressive muscle wasting and low-grade systemic increases in cytokines such as IL-6 and TNF-α. Higher systemic cytokine levels are associated with functional decline and often cachectic disease [7, 93, 109]. Monocytes are involved in skeletal muscle repair through proinflammatory and alternative functions [49, 51]. Przybyla B et al. [88] quantified the total number of macrophages and their pro- and antiinflammatory subpopulations, as well as related cytokine expression, in muscle from young and elderly subjects before and after exercise and found that the number of macrophages within skeletal muscle from the elderly is decreased and their functional properties show defects both at rest and in response to resistance exercise, which could contribute mechanistically to age-related muscle loss [72].

The macrophage lineage displays extreme functional and phenotypic heterogeneity which appears to due in large part to the ability of macrophages to functionally adapt to changes in their tissue microenvironment. This functional plasticity plays a critical role in their ability to respond to tissue damage and/or infection and to contribute to clearance of damaged tissues and invading microorganisms, to contribute to recruitment of the adaptive immune system, and to contribute to resolution of the wound and of the immune response. Ageing alters the proportion and abundance of monocuclear phagocyte subsets. Immune cell functions, including monocyte–macrophage functional plasticity, are known to decrease with age [105].

Evidence has accumulated that environmental influences, such as stromal function and imbalances in hormones and cytokines, contribute significantly to the dysfunction of the adaptive as well as innate immune system in the elderly. A current hypothesis is that the age-associated dysfunction of monocyte–macrophages is the result of their functional adaptation to the age-associated changes in tissue environments. The resultant loss of orchestration of the functional capabilities of these cells would undermine the efficacy of both the innate and adaptive immune systems. Both the T-lymphocyte and B-lymphocyte compartments of the adaptive immune system deteriorate progressively with advancing age [34, 35, 64, 84, 108]. The implications of this hypothesis are that mononuclear phagocyte function may change with age in a tissue specific manner, that changes in macrophage function may contribute sig-

nificantly to decreased clearance of microorganisms and decreased responsiveness of the adaptive immune system.

DCs and monocytes are progressively affected during ageing. A numerical reduction of PBDCs concomitant with increase of monocytes and an impaired ability of both populations to produce IL-12 have been documented during senescence. The ability of PBDCs to produce IL-12 upon lipopolysaccharide (LPS) stimulation progressively declines with age, while their ability to produce IL-10 remains unaffected. Monocytes show the same selective impairment. Given the central role of IL-12 in the induction of protective immunity, this finding appears relevant to the increased incidence of morbidity and mortality from infections and cancer occurring in aged people. The decrease in IL-12 production may contribute to the dysregulation between the T-helper (TH)1 and TH2 subsets, characterized by a predominant production of TH2 cytokines, which has been described in the elderly [32, 34, 75].

Influenza virus-specific T-cell responses are decreased in the aged, and it is in part a result of defects in antigen presentation. The increased incidence of pneumococcal infections is a result of a defect in the production of antibodies to the capsular polysaccharide antigens, which are critical for killing of the bacteria by the phagocytic cells. This is in part a result of deficiencies in function [14].

Aged subjects are susceptible to infection with Streptococcus pneumoniaebacteria as a result of an inability to make antibodies to capsular polysaccharides. This is partly a result of decreased production of proinflammatory cytokines and increased production of IL-10 by mononuclear phagocytes. A major reason for the inability of macrophages from the aged to support B-cell responses to polysaccharide antigens is a result of a defect in secretion of IL-1 and IL-6. However, the cytokine secretion defect is not limited to IL-1 and IL-6, as other proinflammatory eytokines, such as IL-12 and TNF-α are also produced at lower levels by macrophages from the aged in comparison with young. To understand the molecular basis of cytokine dysregulation in aged mouse macrophages, Chelvarajan RL et al. [15] performed a microarray analysis on RNA from resting and LPS-stimulated macrophages from aged and control mice revealing that immune response (proinflammatory chemokines, cytokines, and their receptors) and signal transduction genes were specifically reduced in aged mouse macrophages following LPS stimulation. Accordingly, expression of IL-l and IL-6 was reduced, and IL-l0 was increased. There was also decreased expression of IFN-γ. Genes in the Toll-like receptor-signaling pathway leading to nuclear factor-kB activation were also down-regulated by IL-1 receptor-associated kinase 3, a negative regulator of this pathway. An increase in expression of the gene for p38 MAPK was observed with a corresponding increase in protein expression and enzyme activity confirmed by Western blotting. Low doses of a p38 MAPK inhibitor enhanced proinflammatory cytokine production by macrophages and reduced IL-10 levels, indicating that increased p38 MAPK activity has a role in cytokine dysregulation in the aged mouse monocyte–macrophages [8, 19]. Macrophages from the aged were not defective in IL-10 production but produced more of this cytokine than macrophages from the young. Thus, the cytokine production is dysregulated in monocyte–macrophages from the aged following LPS stimulation.

A reduction in secretion of VEGF and expression of CAMs are thought to contribute to the delay in wound healing in the aged. In contrast, peritoneal macrophages from aged mice have been shown to produce more cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) in response to LPS stimulation. Moreover, the expression of a variety of TLRs, including TLR4, is decreased in the aged, which could be the reason for a decreased response of mononuclear phagocytes from aged mice to LPS [26].

LPS stimulation not only induces expression of many genes but also represses many genes that are constitutively expressed in mononuclear phagocytes [38]. Some of these repressed genes include PPAR-γ, CCL24, and CCRl. PPAR-γ has been shown to inhibit production of several inflammatory mediators such as TNF-α, IL-1, IL-6, and inducible nitric oxide synthase (NOS) and its suppression by LPS may be a prerequisite for the induction of the LPS-induced inflammatory phenotype [103, 104]. Macrophages from aged mice have a global defect in the TLR signaling pathway and in production of proinflammatory cytokines and chemokines, and the antiinflammatory cytokines are increased, such that the splenic macrophages in the aged have an antiinflammatory phenotype.

Kang et al. [105, 106] observed an age-related increase in COX-2 expression in monocytes. Cyclooxygenase catalyses the formation of prostanoids that are crucial in maintaining homeostasis and important in inflammation. The increased COX-2 in monocytes of older humans, which is mirrored in rats, may have downstream implications in atherosclerosis and cardiovascular risk as mononuclear prostanoids are implicated in atherosclerotic plaque stability. COX-2 is the major COX system in monocytes and monocytes-derived macrophages. Upon activation, these cells are responsible for production of COX-2-derived PGE_2, which is an important signaling molecule. Therefore, increased expression of COX-2 may lead to enhanced PGE_2 production, which is known to promote atherosclerotic plaque instability by stimulating MMP-2 and MMP-9 to degrade plaque architecture. It is interesting to note that in ageing rats, monocyte COX-2 expression increase in line with COX-2 levels in vascular smooth muscle and endothelial cells, indicating that these blood elements may be a predictor of systemic status [107].

The mechanism for an age-related change in COX formation is elusive. However, one mechanism could involve histones. When histones are acetylated by histone acetyltransferase, the DNA becomes more accessible to transcription factors. Also, age-linked increases in oxidative stress, proinflammatory agents (IL-1, IL-6), and total cholesterol levels could be involved [60].

There is some controversy concerning the basis for the decline in production of inflammatory cytokines and oxidative radicals in response to LPS stimulation. Renshaw et al.[28, 92] reported that the expression of TLR on macrophages was reduced with advancing age and that this was the basis for the reduced cytokine production upon stimulation with LPS. Boehmer et al. [9, 10] reported that TLR expression was not impacted by advanced age. The influence of aging appears to be selective. Macrophages from aged mice have increased levels of COX-2 and produce elevated levels of PGE2 upon stimulation with LPS [53, 113]. LPS induction of IL-10 production also appears to be elevated in macrophages from aged

rodents and humans [96, 100]. It thus appears that aging selectively impacts LPS-induced signaling cascades such that some functions are depressed and others are elevated. Another example of a signaling deficiency that appears in advanced age is responsiveness to IFN-γ. Although expression of the receptor for IFN-γ appears to be normal, IFNγ-induced phosphorylation of MAPK and STAT-1 is reduced in aged rodents [27, 115]. Oxidative stress is hypothesized to alter transcription factors and nuclear receptors and thus alter the ability of macrophages to respond to inflammatory stimuli [68]. Antioxidants do seem to improve monocyte inflammatory function [87, 101, 114]. Neuroendocrine factors and stress hormones have also been hypothesized to contribute to the immunosenescence and decreased macrophage function. Haynes et al. [54], reported that administration of inflammatory cytokines of the innate immune system enhanced the adaptive immune response of aged mice, so that restoration of the functional balance of mononuclear phagocytes in the elderly will not only improve innate responses but, as a result, improve the function of the adaptive immune system, as well.

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NK and NKT Cells

NK Cells in Human Ageing

Raquel Tarazona, Inmaculada Gayoso, Corona Alonso, M. Luisa Pita, Esther Peralbo, Javier G. Casado, Beatriz Sánchez-Correa, Sara Morgado and Rafael Solana

Contents

Abstract: NK cells are cytotoxic lymphocytes that are involved in the early defense against virus infected and tumor cells. NK cells exhibit the capacity to distinguish normal and damaged cells as well as self- and foreign cells. Besides their cytotoxic capacity NK cells also regulate the immune response by producing cytokines and chemokines that directly participate in the elimination of pathogens or activate other cellular components of immunity. NK cells express a broad range of activating receptors and their function is controlled by inhibitory receptors specific for the MHC class I molecules that are ubiquitously expressed on target cells.

Several alterations have been described in human NK cell function with advancing ageing, therefore contributing to immunosenescence. Thus whereas healthy elderly, including centenarians, have preserved NK cell number and function, a decrease in NK cell activity is associated to increased incidence of infectious and inflammatory diseases and to increased risk of death due to infection. Here, we describe recent data about the effects of ageing on NK cells.

Keywords: Ageing • Immunosenescence • NK cells • Cytokines • NK cell ceptors

R. Solana (✉) · I. Gayoso · C. Alonso · M. L. Pita · E. Peralbo
Department of Immunology
Reina Sofia University Hospital
University of Córdoba, Spain
E-mail: rsolana@uco.es

R. Tarazona · J. G. Casado · B. Sánchez-Correa · S. Morgado
Immunology Unit
Department of Physiology
University of Extremadura, Cáceres, Spain

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1 Introduction

Although it had been generally accepted that some aspects of innate immunity, are well preserved in ageing (Pawelec et al. 1998), cumulative evidences in the last decade support the existence of age-associated changes in the cellular components of the innate immune system, including NK cells, that are important in the increased susceptibility of elderly individuals to infectious diseases (Delarosa et al. 2006; Solana et al. 2006).

2 Natural Killer Cells

Natural killer (NK) cells are bone marrow-derived lymphocytes that participate in the early defense against intracellular pathogens and tumor cells. NK cells are part of the innate immunity arsenal and have been defined as cytotoxic non-T lymphocytes. The most important characteristic that distinguishes T-cells from NK cells is the T-cell antigen receptor (TcR) which is made from rearranging genes and is clonally expressed (Parham 2006). NK cells act within hours of infection in contrast to T-cells that require several days to arise. NK cells are characterized by the expression of CD56, an isoform of the neural cell adhesion molecule (N-CAM) and/or CD16, the low-affinity IgG Fc receptor (FcγRIIIa). The discovery on NK cells of receptors for polymorphic major histocompatibility complex (MHC) class I molecules has contributed to better understanding of NK cell biology. In spite of this, NK and T-cells have much in common: cell-surface molecules, effector functions as cytokine secretion and cytotoxicity. Many of the cell surface molecules we called NK cell associated receptors (NKR) are also expressed by subpopulations of T-cells and NKR expression on T-cells has been associated to memory/effector cells (Tarazona et al. 2002, 2004; Vallejo et al. 2004; Abedin et al. 2005; Casado et al. 2005; Delarosa et al. 2006; Michel et al. 2006; Gayoso et al. 2007; Solana et al. 2007; Lemster et al. 2008).

Although NK cells have been considered for many years as being a simple, homogenous and unspecific population in comparison with T- or B cells of adaptive immunity, different subsets have been defined according to the expression of NK markers and their capacity to kill or produce cytokines. Thus, human NK cells can be divided into two functional subsets based on their cell surface density of CD56, $CD56^{bright}$ immunoregulatory cells and $CD56^{dim}$ cytotoxic cells. Both subsets have been characterized extensively regarding their different functions, phenotype, and tissue localization. The $CD56^{bright}$ NK cell subset has a distinctive role in the innate immune response as the primary source of NK cell-derived immunoregulatory cytokines (Cooper et al. 2001; Farag et al. 2003; Wendt et al. 2006). $CD56^{dim}$ and $CD56^{bright}$ subsets also differ in the expression of chemokine receptors that may contribute to cell trafficking (Cooper et al. 2001; Fehniger et al. 2003; Berahovich et al. 2006).

NK cells were long thought to respond directly to tumor or infected cells, but recent data show that NK cells acquire functionality through priming by dendritic cells (DC; Zitvogel et al. 2006; Long 2007; Lucas et al. 2007). This cross-talk between NK cells and myeloid DC also leads to DC maturation and may determine the quality and strength of the adaptive immunity responses (Vitale et al. 2005; Moretta et al. 2006).

NK cells exhibit the capacity to distinguish normal and damaged cells as well as self- and foreign cells. NK cell function is controlled by inhibitory receptors for the MHC class I molecules that are ubiquitously expressed on target cells (Table 1). In consequence, MHC class I positive targets are more resistant to NK mediated lysis. Human receptors for HLA class I molecules can be included into two structural types, those with immunoglobulin (Ig)-type domains (killer Ig-like receptors (KIR) and leukocyte immunoglobulin-like receptor) and those with lectin-like domains called CD94/NKG2 receptors. Inhibitory and activating forms of KIR and CD94/NKG2 receptors have been described. The ligands for KIRs are polymorphic determinants of HLA-A, HLA-B and HLA-C molecules whereas the ligands for the human CD94/NKG2 receptor are complexes of HLA-E bound to peptides derived from the leader sequences of other HLA class I molecules (Borrego et al. 2002; Lopez-Botet et al. 2004; Lanier 2005; Guma et al. 2006). HLA-G, a non-classical MHC class I molecule, is recognized by Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1/LIR1/ILT2/CD85j) and member 2 (LILRB2/LIR2/ILT4/CD85d) and KIR2DL4 (Shiroishi et al. 2006). Inhibitory receptors play a role in "missing-self" recognition, that confers to NK cells the capacity to attack cells that lose or downregulate the expression of MHC class I molecules. However, the expressions of inhibitory receptors on NK cells is not uniform and are germline-encoded by a set of polymorphic genes that segregate independently from MHC genes. Therefore, how NK cell self-tolerance arises in vivo is still poorly understood.

Licensing of NK cells by self-MHC class I has been proposed as a mechanisms for NK cell tolerance to self. This process takes place during NK cell maturation and involves inhibitory receptors that recognize target cell MHC class I molecules. This process results in two types of tolerant NK cells: functionally competent (licensed) NK cells, whose effector responses are inhibited by self-MHC class I molecules through the same receptors that conferred licensing, and functionally incompetent

Table 1 HLA class I specific inhibitory receptors expressed on human peripheral blood NK cells

Receptor	Ligand
KIR2DL1	HLA-C group 2
KIR2DL2/3	HLA-C group 1
KIR3DL1	HLA-B alleles
KIR3DL2	HLA-A alleles
CD94/NKG2A	HLA-E
KIR2DL4	HLA-G
ILT-2/CD85j	HLA-G and other HLA class I molecules
ILT-4/CD85d	HLA-G and other HLA class I molecules

(unlicensed) NK cells. Although this process has been defined for mouse NK cells several findings suggest that human NK cells also undergo this maturation process termed licensing (Kim et al. 2005; Parham 2006; Raulet 2006; Raulet and Vance 2006; Yokoyama and Kim 2006). Once NK cells acquire functional competence through "licensing'' by self-MHC molecules, the result of effector-target interactions is governed by the integration of inhibitory and activating signals that determines whether the NK cell is finally activated, secretes cytokines and lyses target cells (Gasser and Raulet 2006).

NK cells recognize infected cells or tumor cells by using different types of activating receptors (Table 2) that may act in synergy to enhance cytotoxicity or cytokine release after activation (Bryceson et al. 2006). Activating receptors expressed by NK cells include besides the well characterized receptor CD16 that binds FcγRIIIa, NKG2D, CD244, NKp80 and the natural cytotoxicity receptors (NCR) NKp30, NKp46, NKp44. Ligands for activating receptors comprise both non-self ligands and self proteins up-regulated on damaged cells.

The C-type lectin-like receptor NKG2D is unique among activating receptors in that it recognizes a wide range of ligands some of which are primarily expressed in "stressed" tissues or on tumor cells. Human NKG2D ligands are the MHC class I chain related (MIC) proteins MICA and MICB and the UL-16 binding proteins ULBP-1, ULBP-2, ULBP-3 and ULBP-4 (Eagle and Trowsdale 2007; Mistry and O'Callaghan 2007).

NKp30 and NKp46 are constitutively expressed in NK cells and NKp44 is induced after activation (Arnon et al. 2006; Bryceson et al. 2006; Gasser and Raulet 2006). The NKp46 and NKp44 receptors recognize viral haemagglutinins (Draghi et al. 2007; Ho et al. 2008; Cagnano et al. 2008) and NKp30 has been shown to bind a still undefined ligand on DCs. This binding can be inhibited by

Table 2 Activating receptors expressed on human peripheral blood NK cells

Receptor	Ligand
CD16	IgG
NKp30	Unknown
NKp46	Viral haemaglutinin
NKp44*	Viral haemaglutinin
KIR2DS1	HLA-C group 2
KIR2DS2	HLA-C group 1
KIR2DS3	Unknown
KIR3DS1	HLA-Bw4?
CD94/NKG2C	HLA-E
NKG2D	MICA/B, ULBP1-4
CD244 (2B4)	CD48
DNAM-1	CD155, CD112
CRACC	CRACC
NTB-A	NTB-A

* Induced after activation

the main tegument protein of human cytomegalovirus, pp65 (Arnon et al. 2005, 2006).

Along with CD244, that binds CD48, other members of the signaling lymphocytic activating molecule (SLAM) family of NK cell receptors have been identified: NTB-A and CRACC, which bind NTB-A and CRACC, respectively.

Strong stimulatory signaling resulting from increased levels of stimulatory ligands can often overcome inhibitory signals provided by MHC class I molecules expressed on target cells (Bauer et al. 1999; Cerwenka et al. 2000; Diefenbach et al. 2000).

3 Effect of Ageing on NK Cell Number and Kinetics

Several alterations have been described in NK cells with advancing age, both in animals and humans. In old humans, contradictory data exist due mainly to the different selection criteria of the elderly populations studied, a common problem when comparing studies by different research groups. Thus, whereas there are studies showing that overall NK cell number and cytotoxicity is not significantly affected in very healthy elderly people including centenarians, in other studies that have not used the same strict selection criteria, the number or functions of these cells from elderly subjects are decreased (Table 3).

In a recent study it has been shown that ageing has an impact on NK cell kinetics (Zhang et al. 2007). The analysis of NK cell homeostasis using deuterium-enriched glucose has shown that these cells are in a state of dynamic homeostasis consistent with a model of postmitotic maturation preceding circulation and with a turnover time in blood of about 2 weeks. In young healthy individuals the proliferation rate is 4,3±2,4%/day, equivalent to a doubling time of 16 days, the total production rate is $15\pm7\times10^6$ cells/l/day and the half-life is approximately 10 days. However in NK cells from healthy elderly subjects the proliferation and production rates are significantly lower (2,5±1,0%/day and $7,3\pm3,7\times10^6$ cells/l/day, respectively; Zhang et al. 2007). This study demonstrates that NK cell numbers are well preserved in healthy ageing, in spite of evidences for a reduction in total NK cell production rates of about 50%. These results suggest an increased proportion of long-lived NK cells in the elderly subjects. This may be related to the increased proportion of $CD56^{dim}$ cells, as previously reported in elderly subjects (Borrego et al. 1999).

The decreased proliferation and production rates of NK cells in the elderly can be associated to the telomere shortening observed in the elderly. Thus it has been shown that NK lymphocytes show an age-associated loss of telomeres together with an age-associated reduction of telomerase activity that was evident in individuals over 80 years of age in particular in the oldest individuals and in those with increased NK cell numbers (Mariani et al. 2003a, b).

Table 3 Effect of ageing on the NK cell compartment

	Decreased	Preserved	Increased
Percentage of NK cells			Facchini et al. 1987; Mariani et al. 1994; Borrego et al. 1999; Lutz et al. 2005
Number of NK cells			Borrego et al. 1999; Di Lorenzo G. et al. 1999
CD56 dim subset			Krishnaraj 1997; Borrego et al. 1999
CD56 bright subset	Krishnaraj 1997; Borrego et al. 1999		
Perforin content	Rukavina et al. 1998	Mariani et al. 1996	
Cytotoxicity	Facchini et al. 1987; Mariani et al. 1990; Solana and Mariani 2000; Ogata et al. 2001	Sansoni et al. 1993; Kutza and Murasko 1994, 1996	
Intracellular signaling	Mariani et al. 1998a		
ADCC		Sansoni et al. 1993; Mariani et al. 1998a; Solana and Mariani 2000; Plackett et al. 2004; Lutz et al. 2005	
Response to cytokines	Dussault and Miller 1994; Borrego et al. 1999; Murasko and Jiang 2005		
Cytokine and chemokine production	Mariani et al. 2001 2000a, 2000b; Mocchegiani and Malavolta 2004		
In vivo proliferation and production rates	Zhang et al. 2007		

4 NK Cells and Health Status in the Elderly

An extensive analysis of NK cell number and function in elderly individuals strengthens the significance of NK cell activity in healthy ageing and longevity. Thus a decreased NK cell function in old individuals is associated with an increased incidence of infectious diseases and death due to infection in elderly humans (Ogata et al. 1997, 2001) and elderly people (aged >85 years) with low numbers of NK cells were reported to have three times the mortality risk in the first two years of follow-up than those with high NK cell numbers (Remarque and Pawelec 1998). It has been also reported that decreased NK cell activity in the elderly is also associated with increased frequency of disorders as atherosclerosis

(Bruunsgaard et al. 2001). In a similar way it has been shown that a preserved NK function is related to better health status and lower incidence of respiratory tract infections in elderly individuals and to a better response to influenza vaccination (Mysliwska et al. 2004). Additional evidences supporting the significance of NK cells in healthy ageing come from studies in centenarians, that, in general, have a very well preserved NK cell cytotoxicity (Sansoni et al. 1992, 1993; Franceschi et al. 1995). Furthermore, when NK cells are studied in nonagenarians and centenarians the results show that higher NK cell numbers and NK cytolytic activity were associated with better retained ability to maintain an autonomous life style. These parameters were also associated with higher serum vitamin D levels, a well-nourished status and balanced basal metabolism, indicating the impact of hormonal and nutritional variables on NK cell function in elderly people and again emphasizing that results on NK cells may depend to a much greater extent than T-cells on the state of health of the individual (Mariani et al. 1998b; Pawelec et al. 1998). Moreover, the percentage of NK cells has been shown to correlate with serum zinc and selenium concentrations, and with plasma vitamin E and ubiquinone-10 concentrations, confirming that micronutrients may affect the number and function of NK cells in old age (Mariani et al. 1998b; Ravaglia et al. 2000). This suggests that any analysis of biomarkers of immunosenescence must of necessity take these variables into account.

Together, these results support the fact that preserved NK cytotoxicity can be considered a marker of healthy ageing, whereas low NK cytotoxicity is a predictor of increased morbidity and mortality due to infections.

5 Effect of Ageing on the Expression and Function of NK Cell Receptors

Although the overall NK cell cytotoxicity seems not to be significantly affected in the very healthy elderly donors, it has been demonstrated that, even in these donors, there is a decreased cytotoxicity per NK cell, associated with defective signal transduction (Table 3; Mariani et al. 1998a; Solana and Mariani 2000). Thus, the maintenance of NK cell activity is probably due to a compensatory increase in the number of NK cells to accommodate a possible decrement of NK cell cytotoxicity (Mariani et al. 1994). This increased cell number has been related to a higher number of $CD56^{dim}$ rather than $CD56^{bright}$ subset containing the most cytotoxic NK cells (Borrego et al. 1999; Solana et al. 1999). Neither the binding of effector cells to the target cells nor the perforin content of NK cells is significantly different in the old and young groups. On the contrary the defective NK cell cytotoxicity is associated with a decreased capacity of NK cells to release IP3 after interacting with the target cells and a delayed hydrolysis of PIP2, indicating that the PKC-dependent pathway is affected as a consequence of ageing (Mariani et al. 1998a). However NK activation and cytotoxic granule release induced by CD16 crosslinking is not affected by ageing (Pawelec et al. 1998; Solana et al. 1999; Solana and Mariani 2000; Bruunsgaard et al. 2001; Lutz et al. 2005).

Furthermore the PI-3-kinase pathway coupled to CD16 triggering is not significantly affected in NK cells from elderly people, indicating that the transduction pathways involved in natural or CD16-dependent NK cytotoxicity are differentially affected by ageing (Mariani et al. 1998a; Solana and Mariani 2000).

Despite the maintenance of CD16-mediated killing, the decreased per-cell NK cytotoxicity against the classic target cell line K562 suggests that the expression and/or the functionality of other NK activating receptors are likely to be defective in the elderly. Very little is known about the effects of senescence on the function of NK receptors, and discrepant results have been reported in this context. Whereas it was reported that the expression of HLA-specific killer immunoglobulin-like receptors is not significantly affected in NK cells from elderly compared to young donors (Mariani et al. 1994), other study has shown that NK cells present an age-related increase in KIR expression and a reciprocal decrease in CD94/NKG2A expression, although the CD94/NKG2A inhibitory signaling pathway is intact (Lutz et al. 2005).

In relation with the expression of other NK receptors involved in NK cell cytotoxicity, our results show that NK cells from elderly donors have a decreased expression of the activating receptor NKp30 (Fig. 1). NKp30 mediates the crosstalk between NK and DCs via the recognition of an unknown ligand expressed on DCs. As summarized on Figure 1 the engagement of the NKp30 receptor can lead either to a direct killing of DCs by NK cells, or to the secretion of IFN-gamma and TNF-alpha and the subsequent maturation of DCs. Therefore NK-activated DCs loaded with tumor or virally derived antigen have an increased capacity to prime T-cells. In return, activated DCs release Th1 cytokines that further enhances NK activation (Arnon et al. 2005, 2006). The decreased expression of this receptor on NK cells

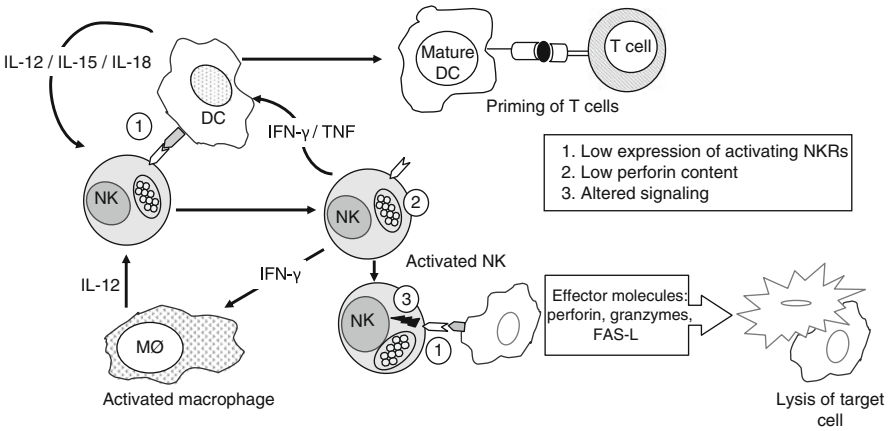

Fig. 1 Effect of human ageing on NK cell function. Cross-talk of NK cells with DCs through NKp30 receptor interaction with its unknown ligand results in inducing DCs maturation and NK cell activation. Whereas DCs collaborate with T-cells in the initiation of adaptive response, activated NK cells produce cytokines and kill target cells. Age-associated alterations in NK cell include: (1) Low expression of activating NKRs that could result in defective cross-talk with dendritic cells and defective recognition of target cells, (2) low perforin content, and (3) altered signal transduction

from elderly individuals should also affect the interaction between these cells leading to a decreased capacity to collaborate in the initiation of the adaptive immune response against virus infected or tumor cells (Fig. 1).

6 Effect of Ageing on NK Cell Response to Cytokines

Cytokine activation of NK cells results in enhanced cytotoxicity and in the synthesis and release of cytokines and chemokines. The enhancement of the cytotoxic activity of NK cells in response to IL-2, IL-12 or IFN-α and γ is well preserved in the healthy elderly. However, the capacity of these cytokine-activated killer cells to lyse the NK-resistant Daudi cell line is significantly decreased in the elderly (Kutza and Murasko 1994, 1996; Murasko and Jiang 2005). A major effect of ageing on cytokine and chemokine production by NK cells is a marked early decrease in IFN-γ secretion in response to IL-2, which can be overcome by increasing the incubation time (Murasko and Jiang 2005). In a similar way the production of MIP-1α, Rantes

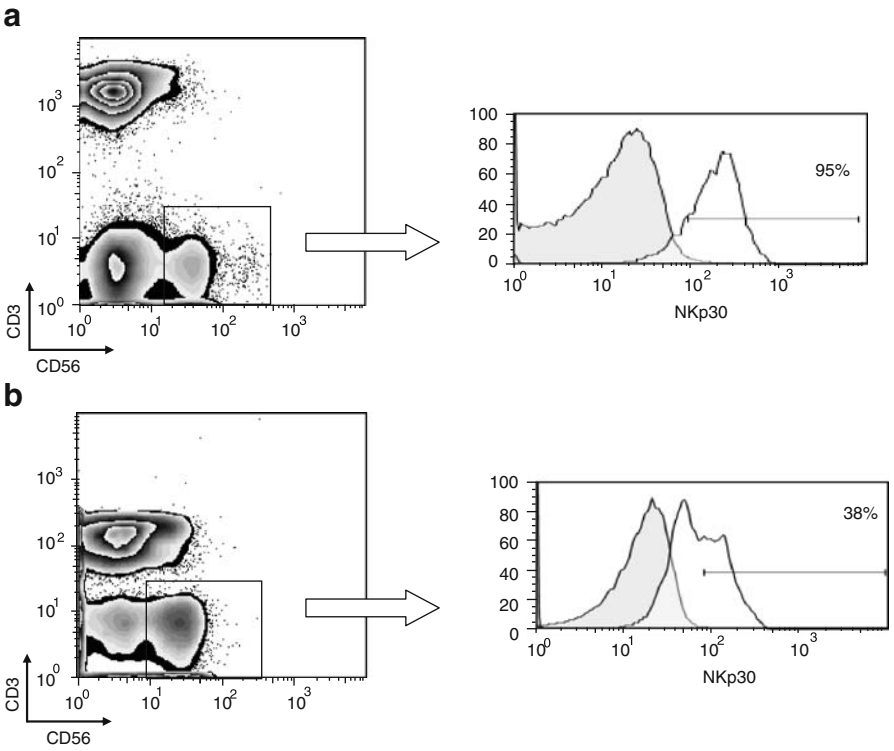

Fig. 2 Expression of NKp30 on NK cells from healthy young (a) and elderly (b) individuals. Peripheral blood lymphocytes were labeled with monoclonal antibodies against CD3, CD56 and NKp30. Results were analysed with a FACSCanto cytometer. Reduction of percentage and mean fluorescence channel of NKp30 was observed in elderly individuals

and IL-8 chemotactic cytokines by NK cells is decreased both in elderly subjects in response to IL-2 and in nonagenarians in response to IL-2 or IL-12 although these cells express the corresponding chemokine receptors. Because of the co-stimulatory role of chemokines on NK cell responses, the decreased production of chemokines can be involved in the defective functional activity of NK cells from old subjects (Mariani et al. 2001, 2002a, b).

Ageing also affects the response of NK cells to IFN-α/β both in mice and humans. This decreased response could be related to the delay in virus clearance observed in aged mice (Murasko and Jiang 2005). These results suggest that NK cells do show an age-associated defect in their response to cytokines, with a subsequent detriment both in their capacity to kill target cells and synthesize cytokines and chemokines.

7 Conclusions and Perspectives

NK cells are a key component of innate immunity in the elimination of virus infected or tumor cells. Recent evidences also support their significance in the initiation of adaptive responses by their crosstalk with DCs and subsequent activation of T-cells. NK cells can be affected by ageing, although several studies have shown a good correlation between the number and/or function of NK cells and the maintenance of an adequate health status in elderly and very elderly people (including nonagenarians and centenarians). On the contrary a decreased NK cell function is associated to increased risk of infectious diseases and risk of death due to infections, supporting the importance of the altered functions of NK cells in the age-associated deterioration of the immune system called immunosenescence.

Our recent finding that NK cells from healthy elderly individuals have a decreased expression of NKp30 receptor, important not only in NK cytotoxicity but also in regulating their cross-talk with DCs strongly support that the alterations in NK cells by ageing may have important consequences that may help to explain the association between a preserved NK cell function and the maintenance of a healthy status. Further studies on the effect of ageing on all NK cell subsets, on the expression and function of activating and inhibitory receptors and a more profound study of the molecular mechanisms involved in these processes are required to better understand the contribution of NK cell ageing to immunosenescence. Considering the increasing advances in the understanding of the mechanisms involved in NK cell interactions not only with tumor and virus infected target cells but also with other cells of the immune system the analysis of how ageing affect these different processes is mandatory.

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Natural Killer Cells and Human Longevity

Hideto Tamura and Kiyoyuki Ogata

Contents

Abbreviations

NK	Natural killer
DCs	Dendritic cells
PB	Peripheral blood
ADCC	Antibody-dependent cellular cytotoxicity
LNs	Lymph nodes
MHC	Major histocompatibility complex
IL	Interleukin
KIR	Killer Ig-like receptor
BM	Bone-marrow
Flt3	Fms tyrosine kinase 3
IFN	Interferon
NKRs	NK cell receptors
HLA	Human leukocyte antigen
MIC	MHC-I polypeptide-related sequence
TLR	Toll-like receptors
HIV	Human immunodeficiency virus
TNF	Tumor necrosis factor
GM-CSF	Granu locyte macrophage colony-stimulating factor
IHLs	Intrahepatic lymphocytes

K. Ogata (✉) · H. Tamura
Division of Hematology
Department of Medicine
Nippon Medical School
1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan
Tel.: +81-3-3822-2131
Fax: +81-3-5685-1793
E-mail: ogata@nms.ac.jp

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HCV	Hepatitis C virus
CMV	Cytomegalovirus
MDS	Myelodysplastic syndromes
LU	Lytic units
PCNK	NK activity on a per-cell basis
PS	Performance status

Abstract: Natural killer (NK) cells are a lymphocyte subset in the innate immune system. These cells not only mount an early immune response to infections and neoplasia but also affect the adaptive immune system by communicating with dendritic cells. In this chapter, we review basic findings on NK-cells and then information from 1) rare patients with isolated NK-cell deficiency, 2) patients with certain malignant neoplasia, and 3) healthy middle-aged and elderly individuals. Those findings indicate that NK-cells are crucial immune components for sustaining life. With increasing age, numbers of T- and B-lymphocytes decline while the number of NK-cells increases. This is especially marked in centenarians. In terms of reduced tolerance to stress such as infections in the elderly, the power of early responders in the immune system including NK-cells may be especially important.

Keywords: Natural killer cells • Longevity • Infection • Neoplasia

1 Introduction

Studies examining healthy people showed that among the various components in the human immune system, natural killer (NK) cells are well maintained throughout life, even in centenarians. This is sharp contrast to the decline in T- and B-cell numbers with increasing age. Therefore, it is hypothesized that well-preserved NK-cell function is essential for longevity. In this chapter, we summarize basic findings on NK-cells, including recent understanding of the interaction between NK-cells and dendritic cells (DCs), and then review information so far obtained on the role of NK-cells in human longevity.

2 Overview of NK–cells

2.1 NK-cell Subsets

NK-cells were originally identified as a population of large granular lymphocytes and once considered to be a homogenous subset of lymphocytes in the peripheral blood (PB). However, NK-cells consist of heterogeneous populations. They can be divided into two subsets by the expression levels of CD56 (neural cell adhesion molecule, NCAM) and the presence or absence of CD16 antigen (FcγRIIIA),

which binds the Fc portion of IgG and mediates antibody-dependent cellular cytotoxicity (ADCC) by binding to opsonized cells [18, 43]. These two subsets, $CD56^{bright}CD16^{-}$ cells and $CD56^{dim}CD16^{+}$ cells, differ in their homing capabilities, i.e., $CD56^{bright}CD16^{-}$ NK-cells largely predominate in the lymph nodes (LNs) and comprise circa 10% of PB NK-cells, as well as in other functions including cytolytic activities, cytokine production and ability to proliferate. It was reported that the $CD56^{bright}CD16^{-}$ subset potently induces cytokine secretion, is a cytokine-responsive NK-cell subset, but has low intrinsic cytotoxicity, whereas the $CD56^{dim}CD16^{+}$ subset has little intrinsic secretory capability, but potent cytolytic activity [18, 56]. These subsets also express different receptors for chemokines, cytokines, and major histocompatibility complex (MHC) Class I ligands [67]. $CD56^{bright}CD16^{-}$ NK-cells constitutively express interleukin (IL)-2 receptors with high and intermediate affinity and increase in response to low doses of IL-2. On the contrary, $CD56^{dim}CD16^{+}$ NK-cells proliferate weakly in vitro in response to high doses of IL-2 [12]. Finally, resting $CD56^{bright}CD16^{-}$ NK-cells are large granular cells and express high levels of the CD94/NKG2 family and very low levels of the killer Ig-like receptor (KIR) family, while resting $CD56^{dim}CD16^{+}$ NK-cells contain numerous cytolytic granules in the cytoplasm and express both KIR and CD94/NKG2 receptor at relatively high levels [39].

2.2 NK-cell Development and Related Cytokines

NK-cells share a common lymphoid progenitor with thymocytes and B-cells [17]. In both humans and mice, early NK progenitors appear to be bone-marrow (BM)-derived $CD34^{+}$ cells, which express receptors for the *fms* tyrosine kinase 3 (Flt3) ligand, c-kit ligand, and IL-2 receptor β chain (CD122) shared with the trimeric receptor for IL-15, and the BM microenvironment is necessary for complete maturation of NK-cells. BM stroma-derived IL-15 in cooperation with c-kit ligand and Flt-3 ligand is a critical factor for the development of mature NK-cells from NK progenitors in the BM [22, 27]. IL-15, which is reported to protect NK-cells from IL-2 activation-induced cell death, is also important for the maintenance of NK-cells [55, 68, 79]. NK progenitors respond to early-acting, stromal cell-derived growth factors such as the c-kit and Flt3 ligands and develop into NK precursors with the $CD34^{+}$IL-2/IL-15R$\beta^{+}CD56^{-}$ phenotype. IL-15 matures these NK precursors into functional $CD56^{bright}$ NK-cells [28]. However, further studies are required to understand the regulation of $CD56^{dim}$ NK-cell differentiation.

IL-21, another cytokine that can bind the common γ-chain shared with IL-2, IL-4, IL-7, IL-9, and IL-15, plays a role in the proliferation and maturation of NK-cells [63]. IL-7 is an early-acting cytokine responsible for the generation of immature $CD56^{bright}$ NK-cells [86]. IL-2 is a growth factor for NK progenitors and mature NK-cells. In addition, IL-2 induces the production of NK effector molecules and enhances the lytic activity of NK-cells. IL-12 and IL-18 are NK-activating cytokines during late NK-cell differentiation and synergistically enhance the cytotoxicity of

and interferon (IFN)-γ production by NK-cells [34, 44]. IL-1 and IL-18 potentiate the effects of IL-12 by upregulating IL-12 receptors on NK-cells.

2.3 NK-cell Receptors

NK-cell receptors (NKRs) can be classified as inhibitory and activating [10, 53]. The MHC Class I-specific inhibitory receptors were first identified in both mice and humans. It is well known that NK-cells are able to lyse MHC Class I-negative tumors and infected cells, which are not recognized by the inhibitory receptors on NK-cells. Several inhibitory types of receptors exist, including the two main groups consisting of the KIR family (KIR2DL, KIR3DL, etc.) and the heterodimeric, C-type lectin receptors CD94-NKG2A/B, which bind to MHC Class I molecules and human leukocyte antigen (HLA)-E, respectively [10, 11]. HLA-E is a nonclassical, Class Ib molecule for which surface expression requires binding of peptides derived from the leader sequences of different HLA Class I molecules. The lack of even a single MHC Class I allele, which is a frequent event in cancer, sensitizes HLA-E to NK-cell cytotoxicity [53].

NK-cell cytotoxicity is also triggered by activating receptors including MHC Class I-specific receptors, i.e., KIR (KIR2DS, KIR3DS, etc.), C-type lectin receptor CD94/NKG2C, and non-MHC Class I-specific receptors such as natural cytotoxicity receptors, NKG2D, leukocyte adhesion molecule, and DNAX accessory molecule-1 (DNAM-1, CD226). NKG2D and DNAM-1 can recognize stress-induced ligands expressed by several tumor cell lines, such as MHC-I polypeptide-related sequence A (MICA), MHC-I polypeptide-related sequence B (MICB), and UL-16-binding protein (NKG2D ligands), and poliovirus receptor (CD155) and Nectin-2 (CD112, DNAM-1 ligands) [15, 64, 66]. Natural cytotoxcity receptors such as NKp46, NKp44, and NKp30, for which the host ligands remain unknown, mediate the lysis of many types of cancer cells. Additional receptors of NK-cell activation also comprise a series of coreceptors including 2B4, NTB-A, and NKp80 coreceptors, CD18/CD11 (β2 integrins), CD2 adhesion molecules, and Toll-like receptors (TLR) [51, 72]. NK-cell activation depends on the expression of these ligands on the target cells. These receptors provide both inhibitory and activating signals, and the balance between them determines NK-cell activation, proliferation, and effector functions.

2.4 NK-cell Function

The main function of NK-cells is host defense against tumors and infections. NK-cells can directly kill infected cells or tumor cells that have lost the expression of MHC Class I molecules. NK cytotoxicity can be triggered by viral and bacterial products directly binding to surface TLR3 and TLR9 [52, 72]. NK-cells also act as

the conductor for the activation of the immune defense along with T- and B-cells, macrophages, and immune effector cells in local sites.

NK-cells can respond to infections directly by recognizing infected cells, and indirectly by cytokine secretion and interaction with DCs expressing TLR. It has been reported that NK-cells play an important role in antiviral defense, especially in controlling the severity of Herpes virus, Hepatitis, and Human immunodeficiency virus (HIV) infections [7]. To perform this role, NK-cells require the activation of multiple effector pathways including direct cytotoxicity and the release of cytokines and chemokines. Viral infection immediately induces macrophages to produce cytokines such as tumor necrosis factor (TNF)-α, IL-12 and IFN-γ. Activated NK-cells can also secrete several cytokines, i.e., IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), M-CSF, TNF-α, IL-5, IL-10, and IL-13, to control the growth and spread of pathogens and tumors. Furthermore, many of the cytokines produced by NK-cells can affect the initiation and maintenance of adaptive immune responses. Although NK-cells are activated and kill virus-infected cells immediately, it takes 1-2 weeks after infection to activate adaptive immune responses, such as pathogen-specific killer T-cells and antibodies produced by B-cells.

Intrahepatic lymphocytes (IHLs) contain 37% NK-cells and that percentage in the IHL pool may increase to 90% in hepatic disease [23]. Infection with hepatotropic viruses such as Hepatitis C virus (HCV) activate liver NK-cells that play a crucial role in the recruitment of virus-specific T-cells to the liver and in inducing antiviral immunity in the site. Activated NK-cells can kill virus-infected cells by a cytolytic mechanism via the perforin/granzyme and FasL pathways and produce proinflammatory cytokines that can induce an antiviral state in host cells. Compromised NK-cell functions have been reported in chronic HCV-infected patients. To control mouse cytomegalovirus (CMV) infection, NK-cells use two main effector mechanisms: the secretion of IFN-γ and direct lysis of infected cells by exocytosis of granules that contain perforin and granzymes. TLR9 recognizes unmethylated CpG DNA, a component of bacterial and viral DNA, and delivers signals for cellular activation through the adaptor protein MyD88. TLR9-deficient or MyD88-deficient mice show an increased susceptibility to mouse CMV, indicating an important role of TLR9 and MyD88 in protection against mouse CMV [3, 20, 42, 74]. In human CMV infection, activated NK-cells produce IFN-γ and secrete lymphotoxin-α and TNF, which contributes to the NF-κB-dependent release of IFN-β from infected cells. IFN-γ and IFN-β work together to inhibit CMV replication [38].

NK-cell-produced IFN-γ might contribute to protecting humans from Influenza A and Sendai viruses. Contact between NK-cells and virus-infected macrophages induces IFN-γ production. Furthermore, the expression of MICB, a ligand for the NKG2D receptor, was up-regulated in virus-infected macrophages, suggesting the role of MICB in the activation of the IFN-γ gene in NK-cells [71]. In HIV infection, the number of $CD3^{-}CD56^{+}$ NK-cells in the PB was dramatically reduced in patients with ongoing viral replication compared with uninfected or aviremic patients. Therefore, NK-cells play an important role in controlling HIV infection. NK-cells obtained from viremic patients produce more IFN-γ and TNF-α than NK-cells from

aviremic patients [2]. A recent study has shown that the decrease in MHC Class I molecules on T-cell blasts infected with certain HIV strains was selective. The expression of HLA-A and -B was decreased in infected cells, whereas HLA-C and -E remained on the surface. HLA-C and -E bind to the KIR and CD94-NKG2A receptors, respectively, on NK-cells, resulting in inhibition of NK-cell-mediated killing of HIV-infected cells [8].

NK-cells are also activated during parasitic and bacterial infections. NK-cells produce IFN-γ in response the infection of red blood cells with *Plasmodium falciparum*, the causative agent of malaria [4]. The importance of NK-cells in protecting against bacterial infection has been controversial and may depend upon the site of infection or type of inflammatory response elicited. The expression of the activating receptors NKp30, NKp46, and NKG2D was enhanced in NK-cells after exposure to monocytes infected with the intracellular pathogen *Mycobacterium tuberculosis*. The infected monocytes upregulated the expression of the NKG2D ligand ULBP1 through TLR2 activation, and NK-cells lysed infected monocytes through NKG2D- and NKp46-dependent mechanisms [75].

2.5 Localization and Trafficking of NK-cells

NK-cells comprise approximately 5–20% of lymphocytes in the spleen, liver, and PB, and are present at lower levels in the BM, thymus, and LNs [31, 45]. Although NK-cell trafficking is not understood in detail, it was reported that chemokine secretion and chemokine receptor expression by NK-cells are dynamically regulated, and that some chemoattractants and chemokines can induce the migration of NK-cells to inflammation sites [67]. It is possible that changes in key adhesion molecules may induce the physical movement of NK-cells into sites of infection. $CD56^{bright}CD16^{-}$ NK-cells express CCR5 and CCR7 as well as L-selectin, which can attract T-cells to LNs, and $CD56^{dim}CD16^{+}$ NK-cells express CX3CR1. In addition, both of these NK subsets express CXCR3 and CXCR4 [13, 32, 67].

2.6 NK-DC Interactions

The crosstalk between NK-cells and myeloid DCs leads to NK-cell activation and DC maturation. Activated NK-cells can kill DCs that fail to undergo proper maturation; this phenomenon is called “DC editing” [53]. In vitro studies showed that NK-cells activated by IL-2 can kill immature DCs by ligation between NK-activating receptors, mainly NKp30 on NK-cells, and still unidentified cellular ligands on DCs [30, 65]. Consistent with these data, NK-cells derived from patients with acute myeloid leukemia, who frequently exhibit downregulation of NKp30 surface expression, have impaired killing of immature DCs [19, 29]. Furthermore, the NK-cell-mediated DC killing was inhibited by transforming growth factor-β, which can downregulate the

surface expression of NKp30 [14]. Recently, DNAM-1 has been shown to cooperate with NKp30 in the NK-cell-mediated lysis of DCs [64]. The expression of Nectin-2, one of the DNAM-1 ligands, is increased on immature DCs. Studies using immunohistochemistry and confocal microscopy showed that DNAM-1 ligands are expressed by DCs present in normal LNs. In general, the function of activating NKRs is under the control of inhibitory NKRs specific for HLA Class I [54]. However, immature DCs do not follow this rule. Analysis of NK clones showed that an NK subset, which lacks KIR specific for HLA Class I alleles and expresses HLA-E-specific CD94/NKG2A receptors (the $KIR^{-}NKG2A^{dull}$ phenotype), can kill immature DCs [21]. The DC editing mediated by NK-cells might be important in the selection of appropriate DCs in conjunction with the removal of DCs that fail to perform optimal antigen presentation and T-cell priming. NK-cells are not able to kill mature DCs, mainly because mature DCs express higher levels of HLA-E than immature ones.

During NK-DC interaction, NK-cells can induce DC maturation mediated by TNF-α and IFN-γ, which are released upon engagement of the NKp30 triggering receptor [77]. Semino et al showed that NK-DC interaction results in IL-18 secretion by DCs and then IL-18-activated NK-cells secrete the proinflammatory cytokine high mobility group B1 (HMGB1) [70]. HMGB1 can induce DC maturation and protect DCs from lysis. These data suggest that NK-cells mediate DC maturation by several pathways. NK-cells kill tumor cells and virus-infected cells, and subsequently prime DCs with the killed cell-derived antigens to induce specific $CD8^{+}$ T-cell responses [49, 50, 87]. After antigen uptake, DCs undergo maturation and release several cytokines, including IL-12, that enhance NK functions. NK-DC interactions also induce primary tumor rejection and long-term cytotoxic T-lymphocyte memory, bypassing the requirement for $CD4^{+}$ helper T-cells [1].

3 Contribution of NK-cells to Human Longevity

To gain insight into role of NK-cells in human longevity, we review findings 1) from patients with isolated NK-cell deficiency, 2) on the relationship between NK-cells and the development and progression of malignancies in humans, and 3) on NK-cells in the healthy elderly.

3.1 Isolated NK-cell Deficiency in Humans

Various isolated defects in human immune system have been reported, which provide valuable information on the function and importance of each component of the immune system [57]. However, primary isolated NK-cell deficiency, in which other immunologic functions are normal and which occurs in the absence of other immunocompromising conditions, is very rare. Moreover, in some reported cases, the distinction between NK-cells and T-cells expressing NK-cell markers was unclear. The

rarity of isolated NK-cell deficiency may imply the critical role of NK-cells in human life. Another explanation is that because NK-cells are an early responder in the immunogic defense system and communicate with late components in the immune system, another immune component(s) is often secondarily impaired in isolated NK-cell deficiency. In this chapter, we do not discuss patients in whom both NK-cells and other immunlogic components were compromised or unexamined [46, 82].

A girl reported in 1989 by researchers at the University of Massachusetts Medical School is probably the best-known patient with isolated NK-cell deficiency [6]. She had experienced recurrent otitis media since infancy and at the age of 13 years developed severe varicella infection and polymicrobial sepsis with *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* infection. Four years later, she again developed sepsis with *S. aureus* infection and interstitial pneumonia due to CMV. $CD56^+$ and $CD16^+$ cells were completely absent in her PB, along with an almost complete absence of NK-cell cytotoxicity and ADCC. She later developed aplastic anemia and died after undergoing stem cell transplantation [62]. The second patient was a 23-year-old woman who had recurrent condylomata due to human papilloma virus [5]. Her peripheral blood lacked NK ($CD3^-CD56^+$) cells but showed an increase in $CD3^+CD56^+$ cells. NK cytotoxicity was almost completely absent, which was only slightly augmented by the administration of IL-2. However, this patient appeared not to have experienced devastating infections. The third patient was a 5-year-old girl, who experienced repeated otitis media and Herpes virus infections, requiring acyclovir prophylaxis for the latter [40]. Examination of her PB showed a profoundly reduced number of NK ($CD3^-CD56^+$) cells and reduced NK cytotoxicity but normal ADCC. The fourth was a 2-year-old girl with recurrent, ultimately fatal, varicella infection [25]. Her PB also contained a markedly decreased number of NK ($CD3^-CD56^+$) cells and showed reduced NK cytotoxicity.

Another group of patients reported in the literature had a normal number of NK-cells but defective NK-cell functions. Two brothers (6 and 12 years of age), who experienced recurrent upper respiratory tract infections and otitis media, had nearly normal numbers of $CD56^+$ and $CD16^+$ cells in their PB, but NK cytotoxicity was almost completely absent, which was not improved by incubating cells with IL-2 or IFN-α [41]. A recent report has suggested that overexpression of inhibitory killer receptors is a possible underlying mechanism in such patients [33].

Information from the above patients indicates that NK-cells are important in the in vivo defense against viruses mainly in the family Herpesviridae. In patients who lack NK-cells, infections with Herpes viruses are usually life-threatening. Even in healthy individuals, an initial Herpes virus infection may be followed by latency with subsequent reactivation and, in some cases, cause malignancies including lymphoma, nasopharyngeal cancer, and Kaposi's sarcoma. Therefore, it is concluded that NK-cell deficiency in humans is associated with life-threatening diseases caused by Herpes viruses and therefore affects life span. The association between NK-cells and other viral and bacterial infections in vivo is less clear. All four reported patients who lacked NK-cells were women. Further accumulation of such patients and research on their pathophysiology including genetics will be important to understand this association.

3.2 Relationship between NK-cells and the Development and Progression of Malignancies

NK-cells may contribute to human longevity by controlling neoplastic cells in patients with malignancies. An example of data supporting this hypothesis comes from studies of myelodysplastic syndromes (MDS). MDS are malignant disorders of hematopoietic stem cells and predominantly occur in the elderly [58]. A well-designed study in the UK found that in the population aged 70 years and older, more than 50 new MDS patients are diagnosed annually per 100,000 persons [84]. MDS are composed of various subtypes and can be grouped into early-stage and advanced-stage MDS based on the percentage of neoplastic myeloblasts in the BM and PB. It is also believed that early-stage MDS is often overlooked due to the absence of specific signs and symptoms of the disorder [58]. A substantial proportion of patients with early-stage MDS progress to advanced-stage MDS and then to acute myeloid leukemia by mechanisms that have not been thoroughly clarified. MDS patients often have dysfunction in a variety of immunologic components including NK-cells [35]. NK-cell cytotoxicity, which may or may not be stimulated by IL-2 in vitro, is preserved in early-stage MDS but decreases with disease progression [24, 60]. The elevation in levels of circulating soluble IL-2 receptor [85], which can neutralize endogenous IL-2, and reduced expression of activating NK receptors such as NKG2D in NK-cells [24] may contribute to the reduced NK-cell cytotoxicity in advanced-stage MDS patients. These data suggest that NK-cells contribute to controlling disease progression in MDS and thus may affect longevity because of the high prevalence of MDS in the elderly. Similarly, NK-cells may prolong the life span of patients with other cancers by inhibiting disease progression [16, 37, 76].

Meanwhile, it is known that NK-cell activity varies significantly among healthy individuals. The question is whether differences in NK-cell activity in healthy people are involved in the development of malignancies and thus contribute to longevity. Imai et al examined NK-cell cytotoxicity in 3625 healthy Japanese, mainly in the 40-69-year-old age-group, living in Saitama prefecture [36]. They followed the cohort for 11 years to investigate cancer incidence and death from any cause. They recorded 154 cases of cancer (most frequent sites were the stomach, lung, and intestine) in the study period and found that reduced NK-cell cytotoxicity was a statistically significant risk factor for the development of cancer.

3.3 NK-cells in the Healthy Elderly

NK-cells in the healthy elderly have been examined in many studies. Sansoni et al carefully selected 138 healthy individuals, ranging in age from 4 to 106 years, and examined their immunologic parameters including lymphocyte subsets and NK-cell cytotoxicity [69]. They found that although the number of T- and B-cells

declined with increasing age, the number of cells with NK markers did not undergo an age-related decline. Instead, the number of $CD16^+$ cells and $CD57^+$ cells increased with age. Moreover, centenarians had well-preserved NK-cell activity expressed in lytic units (LU), the magnitude of which was comparable to that in healthy young people and higher than that in the healthy middle-aged.

We also selected 82 healthy individuals, aged 30 to 99 years, and investigated their immunologic parameters [61]. We confirmed that the number of T-cells declined and the number of $CD56^+$ cells increased with age and that NK-cell activity expressed as LU was maintained throughout this age range. In addition, because the number of total lymphocytes was found to decrease with age in most studies including ours, we calculated the index of absolute in vivo NK-cell activity (ALU = LU x mononuclear cell count per microliter of PB) and found that the ALU decreased as age increased. Moreover, the cytotoxic activity exerted by one NK-cell (NK activity on a per-cell basis, PCNK) decreased as age increased. When we retrospectively examined the medical records of the elderly in our cohort, it was found that low ALU and PCNK values correlated with a past history of severe infection. Therefore, we proposed that human NK-cells do not escape the aging process and that low NK-cell function is corrrelated with the development of severe infections, which may be fatal, in the elderly. Similarly, several other studies of the healthy elderly showed that the T-cell population declines while the NK-cell population increases in the PB and that the PCNK decreases [9, 26, 47, 48]. In particular, data from centenarians are striking. Miyaji et al reported that roughly 50% of lymphocytes were $CD56^+$ cells in centenarians in contrast to about 11% in the middle-aged [48]. However, it has not been fully clarified whether the NK-cell increase in the very old indicates that NK-cells result in longevity or longevity resulting from other factors causes an increase in the number of NK-cells.

Based on the above findings, we conducted a prospective study to examine whether differences in NK-cell function among the healthy elderly is related to the development of infection and infectious death [59]. Our subjects were 108 immunologically normal elderly people aged 63–99 (mean 81) years residing in nursing homes due to impaired performance status (PS). We determined counts of lymphocytes, monocytes, and neutrophils, serum albumin value, percentage and absolute number of various lymphocyte subsets ($CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, $CD56^+$, $CD3^+HLA{\cdot}DR^+$, $CD3^+CD56^+$, and $CD3^-CD56^+$ cells), and NK-cell activity. The interassay variation in NK-cell activity was minimized by examining the same control cells in each assay. We then followed the cohort and analyzed the correlation between the development of infection during the first 12 months of follow-up and the predetermined parameters as well as age and PS. Using univariate logistic regression analysis, poor PS, low albumin value, old age, and low NK-cell activity correlated significantly with the development of infection. Multivariate logistic regression analysis showed that low NK-cell activity, poor PS score, and older age were independent variables associated with the development of infection. The odds ratio for the development of infection increased

with the decrease in NK-cell activity. We next analyzed correlations between the predetermined parameters and the time until death due to infection in the 108 individuals. Eleven died of infection (all due to pneumonia) during the follow-up period. Univariate Cox proportional-hazards regression analysis showed that poor PS, high $CD8^+$ T-cell count, and low NK-cell activity correlated significantly with short survival time due to infection. Multivariate Cox proportional-hazards regression analysis showed that low NK-cell activity was an independent variable associated with short survival time due to infection. Other independent variables for short survival due to infection were poor PS and a high $CD8^+$ T-cell count. These findings support the hypothesis that well-preserved NK-cell activity is important for human longevity, at least in part because of its antiinfectious effect. The association between a high $CD8^+$ T-cell count and short survival time after infection deserves further discussion. A previous study also suggested an association between a high $CD8^+$ T-cell count and high mortality rate in the elderly [83]. One proposed explanation for this association is that clonal expansions of $CD8^+$ T-cells, which are often observed in the healthy elderly [78], are exaggerated in those with a high $CD8^+$ T-cell count. This clonal expansion probably reduces the naive repertoire of $CD8^+$ T-cells, which impairs T-cell responses and thus may be associated with vulnerability to infections.

Meanwhile, a functional relationship exists between NK-cells and T-cells. In a typical viral infection, NK-cell responses against the virus are observed during the first 1–3 days of infection [80]. These are gradually replaced with viral antigen-specific T-cell responses. When T-cell responses do not occur, as in severe combined immunodeficient mice and athymic nude mice, the increased NK-cell response is maintained for a prolonged period to defend the host [73, 81]. Considering the functional link between NK- and T-cells and the reduced T-cell count commonly observed in elderly people, the role of NK-cells in protecting against infections may be more important in the elderly than in younger individuals.

4 Concluding Remarks

NK-cells are a lymphocyte subset in the innate immune system which are early responders to infections and neoplasia. Data from isolated NK-cell deficiency, certain patients with malignant neoplasia, and healthy individuals all indicate that NK-cells are crucial immune components for sustaining life. In middle-aged people in developed countries who can overcome common infections, NK-cells may be important for defending against malignancies. In elderly people, common infections can cause significant morbidity and mortality. In terms of weakened T- and B-cell immunology as well as reduced tolerance of stress such as infections in the elderly, the strength of the early immune system response including that of NK-cells appears especially important. The role of NK-cells throughout the human life span should be studied further to confirm this hypothesis.

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The Effects of Age on CD1d-restricted NKT-cells and Their Contribution to Peripheral T-cell Immunity

Douglas E. Faunce and Jessica L. Palmer

Contents

1 Introduction

Natural Killer T (NKT) cells are innate lymphocytes known for their roles in regulation of immune responses in cancer, autoimmunity, bacterial and viral infections, and the induction of immunologic tolerance [1–4]. Recently, our laboratory and others have also identified crucial roles for NKT-cells in the regulation of the host response to injury and sepsis [5–7]. As we will discuss further in this chapter, NKT-cells are now widely accepted as critical players in the initiation of maintenance of host defense, as they are uniquely poised to modulate multiple aspects of protective immunity. NKT-cells fill this position via their ability to rapidly produce significant quantities of immunomodulatory cytokines very early during the course of the immune response and can thereby influence the outcome of both innate and adaptive immune processes.

While a significant number of studies, described in this handbook and elsewhere, have identified both direct and indirect effects of advanced age on T-cells, B-cells, and cells of the innate immune system including macrophages, dendritic cells, granulocytes, etc., little is known of how NKT-cell populations might change with age and moreover, how the aging immune microenvironment affects NKT-cell function. Here, we will provide a brief overview of NKT-cells, their role in host defense, and review the limited information on the effects of age on NKT-cell biology.

D. E. Faunce (✉) · J. L. Palmer
Loyola University Medical Center
Bldg 110, Rm 4236
2160 South First Avenue
Maywood, IL 60153, USA
Tel: 708-327-2663,
Fax: 708-327-2813
E-mail: dfaunce@lumc.edu

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2 NKT-cell Development and Restriction by CD1d, Antigen Specificity, and Tissue Distribution

Like virtually all T-lymphocytes, NKT-cells arise and differentiate from mainstream thymic precursors. Whereas conventional $CD4^+$ and $CD8^+$ T-cells differentiate and undergo negative and positive selection based upon thymic expression of self-peptide antigens and of MHC-II and MHC-I, NKT-cells on the other hand, acquire their differentiation signals and undergo thymic selection based upon thymic expression of self-lipid ligands presented on the MHC-I-like molecule, CD1d. NKT-cells arise from $CD4^+$ $CD8^+$ thymic precursors and express a canonical invariant $\alpha\beta$ TCR ($V\alpha14$-$J\alpha18$ in mouse and $V\alpha24$-$J\alpha18$ in human) that recognizes a self-lipid called isoglobotrihexosylceramide (iGb3), which is a breakdown product of the hexosaminadase-B pathway and is presented in the context of CD1d molecules expressed on thymic epithelia [8–10]. Upon engagement of the $V\alpha14$-$J\alpha18$ TCR with iGb3/CD1d complex, double positive NKT precursors down-regulate their expression of CD8. While some NKT precursors retain their expression of CD4 molecules, others eventually down regulate CD4 and become double-negative, invariant TCR-positive. As NKT-cells are so named, they acquire expression of NK lineage receptors including NK1.1, NKG2A/D, Ly49C/I in mouse and CD16, CD56, and CD161 in human.

NKT-cells are widely distributed throughout the body in both humans and mice and can be identified in the thymus, liver, spleen, lymph nodes, and circulation by either their co-expression of the invariant TCR and the above-mentioned NK associated markers or by their ability to bind lipid ligand-loaded CD1d tetramers or dimers [11, 12]. Overall, NKT-cells comprise approximately 0.5–1.0% of the entire T-lymphocyte pool. In the liver, they account for approximately 25–50% of lymphocytes (depending upon species, strain, etc.), in spleen they comprise approximately 2–3% of the lymphocyte population, while in the circulation and lymph nodes, NKT-cells make up only about 0.5–1.0% of circulating lymphocytes. Within the lymphoid compartment NKT-cells can be found in the splenic marginal zones, red pulp, PALS, and paracortical areas [13] (and Faunce et al, unpublished observations) and in the liver, they mainly accumulate in the liver sinusoids. Interestingly, the liver seems particularly adept for the recruitment and retention of NKT-cells, since they constitutively express CXCR6, the receptor for the chemokine CXCL16, which is expressed among other places on the surface of liver sinusoidal epithelium [14]. NKT-cells may also home to lymphoid organs or other sites of inflammation and immune responses via signals mediated through CCR1, CCR2, CCR4, CCR5, CCR6, and CXCR2 [13, 15].

3 CD1d Molecules and Lipid Antigens

Unlike conventional $CD4^+$ and $CD8^+$ T-cells that exhibit specificity for peptide antigens presented by MHC-II and MHC-I respectively, NKT-cells recognize glycolipid antigens presented in the context of the MHC-I-like molecule, CD1d

[1, 3, 16–18]. The CD1 family of cell surface glycoproteins is expressed by a variety of cell types, however, the CD1d isoform is expressed primarily on professional antigen presenting cells including macrophages, dendritic cells and B-cells and is expressed at the cell surface in conjunction with β2-microglobulin. In humans, five isoforms of CD1 exist, CD1a–e. In mice and rats however, only CD1d is expressed [1, 3, 16–18]. The invariant Vα14 (and Vα24) TCR only recognizes CD1d and not the other isoforms and it was the observation in the mid to late 1990's that minor T-cell subsets appeared restricted by CD1d for both function and development that led to the discovery that NKT-cells were indeed a unique subset with their own developmental restriction and antigen specificity [19–22]. Today, the CD1d-restriction of NKT-cells is exploited through the use of CD1d tetramers and dimers that are used for the specific recognition and identification of NKT-cells in both mice and humans [11, 12, 23–25].

As mentioned above, CD1d-restricted NKT-cells exhibit specificity for glycolipid antigens, rather than peptide antigens. The concept that CD1d presents lipid antigens to invariant TCRs was first considered when it was observed that the binding grooves of CD1d as well as the invariant TCR were extremely hydrophobic [20]. The first glycolipid identified as a specific activator of NKT-cells was alpha-galactosylceramide, a lipid isolate of the marine sponge *Agales mauritianus,* whose synthetic analogue KRN7000, exhibited potent anti-tumor immunity mediated by NKT-cells [24, 26–28] and is now known as a potent stimulator for NKT-cells both in vivo and in vitro [8,29–31]. More recently, it has been shown that specific microbial-derived lipids also are presented by CD1d to the invariant TCR for the activation of NKT-cells, including *Sphingomonas* GSL-1 [32], *Borrelia burgdorferi* alpha-glactosyldiacylglycerols [32], and mycobacterial phosphatidylinositolmannosides such as PIM-4 [33]. In addition to reactivity towards exogenous glycolipid antigens presented by CD1d, it is also well established NKT-cell development is restricted by thymic CD1d expression, thereby suggesting the requirement of a self-glycolipid during positive selection of NKT-cell precursors. Indeed, the glycosphingolipid isoglobotrihexosylceramide, or iGb3, appears to be a self-derived glycolipid ligand that is required for positive selection and expansion of NKT-cell precursors during development [10, 34], since β-hexosaminadase deficient mice, which as a result of this enzyme deficiency are unable to convert iGb4 to iGb3, almost completely lack invariant NKT-cells [34].

4 Effects of Age on NKT-cell Numbers and CD1d Expression

Clearly, age-related alterations in the number and/or function of NKT-cells could greatly influence the quality of the effector T-cell response. For the purposes of this chapter, we shall consider studies that have examined NKT-cell numbers and function in humans aged 59 and older and studies in mice of ages twelve months or older. Only a handful of reports have closely examined CD1d-restricted NKT-cell numbers in aged mice and humans and most agree that as age increases, so does the

number of NKT-cells in the periphery [6, 35, 36]. It is not entirely clear whether the increase represents an accumulation of cells over time versus increased expansion of newly made cells. Increased frequency and numbers of NKT-cells could also result from increased recruitment or retention. In fact, it was recently reported by Berzins and colleagues that NKT-cells are comparatively long-lived (i.e., greater than one year in mice) and are retained for significantly greater periods of time in the thymus compared to conventional T-cells [37]. Some reports describe an increase in accumulated, longer-lived hepatic NKT-cells over time in both humans and mice [35, 36]. In mice, NKT-cell number increases 2-to-3-fold in the livers, spleens, and lymph nodes of aged animals [6, 38, 39]. It could be argued that older mice are slightly larger physically and therefore might have greater numbers of cells in general, however, the relative frequency of NKT-cells as compared to conventional T-cells is also 2-to-3-fold higher in aged mice as compared to young [6]. This increase could represent an unusual effect of aging, as most immune cell populations remain static or decrease in number. Some data suggests that this age-related increase in NKT-cells actually stems from newly made cells. Using 5'-bromo-2-deoxyuridine (BrdU) labeling, ligand-loaded CD1d dimer staining and flow cytometry, our laboratory observed that the spleens and lymph nodes of aged mice (18–22 months old) contained nearly 2.5-fold greater numbers of BrdU-positive NKT-cells (Palmer and Faunce, unpublished observations). BrdU is a thymidine analog that incorporates into the newly synthesized DNA, making it an effective marker to distinguish and track newly made cells. The precise mechanisms responsible for increased output of NKT-cells in aged mice remain to be elucidated. Likewise, the reasons for the potentially greater longevity of thymic NKT-cells also require further investigation.

While the majority of observations support the concept that NKT-cells increase with age, as with most topics, the opposite has been reported in that numbers of Vα14 and Vα24 NKT-cells were found to be decreased with advanced age, particularly in the liver [40, 41].

Since NKT-cells are stimulated by lipid ligands presented by the CD1d molecule on APCs, it follows that part of the aged-related breakdown could be attributed to transitions in CD1d expression or magnitude. However, CD1d magnitude and frequency of expression is similar on F4/80$^+$ monocyte/macrophages and CD11c$^+$ dendritic cells in aged and young mice [6]. This suggests that lipid antigen presentation to NKT-cells is intact in aged animals, so changes probably lie within altered responses from NKT-cells once they are activated by ligand-bearing APCs.

5 Aging, NKT-cell Function, and Peripheral T-cell Immunity

Few studies have directly addressed the effects of age on NKT-cell biology, however the topic has been briefly reviewed by others in the recent past [36, 42]. Similarly, very few studies have made direct comparisons of CD1d-restricted NKT-cell involvement in immune function between young and aged subjects, however based on what is known (again considering mice twelve months or older), it appears that NKT-cells

modulate several aspects of T-cell function differently in aged mice compared to young. Recent studies by our laboratory demonstrated that in aged mice, NKT-cells contributed to the age-associated reduction of antigen-specific T-cell proliferation [6]. Removal of NKT-cells (Ly49C$^+$ NK/NKT) from splenic T-cell preparations prior to stimulation restored the capacity of T-cells to proliferate in response to CD3ε ligation. The same observation was made for proliferation of peripheral lymph node T-cells. Importantly, comparable T-cell proliferation in aged and young mice could also be achieved among splenocyte preparations from young vs. aged mice given anti-CD1d monoclonal antibody systemically to block NKT-cell activation in vivo. Such observations implicated NKT-cells in the age-related suppression of the T-cell proliferative response to antigen. Perhaps the most compelling evidence for a direct connection between activated NKT-cells and suppression of T-cell effector function was demonstrated in a series of experiments conducted by our laboratory that utilized delayed type hypersensitivity (DTH) responses after immunization with ovalbumin in complete adjuvant as an index of antigen-specific effector T-cell response in vivo. From studies by our laboratory and others, it is known that aged mice exhibit blunted immune responses (including DTH) in vivo [6, 42–46]. However, we observed that while aged mice given control IgG mounted DTH responses that were 30–50% less in magnitude compared to young mice, aged mice treated systemically with anti-CD1d antibody to block the activation of NKT-cells in vivo, generated DTH responses that were remarkably similar to those seen in young mice [6]. Delayed-type hypersensitivity requires adequate generation of CD4$^+$ effector T-cells during the priming phase in order for the effector arm of the response to proceed. Although aged mice and humans are known to possess fewer CD4$^+$ T-cells and a contracted effector T-cell repertoire, our studies suggested that despite fewer CD4$^+$ T-cell numbers overall, aged mice could generate adequate effector T-cell immunity in vivo when challenged with antigen and an adjuvant, provided NKT-cell activation was attenuated. The idea that blunted effector CD4$^+$ T-cell immunity observed with aging can be overcome is also supported by studies from Haynes and colleagues that used a combination of adjuvant and inflammatory cytokines to achieve results similar to ours [44]. Taken together, the current observations suggest that the decline in the quality of protective T-cell immunity associated with aged animals may be due at least in part, to a CD1d-NKT dependent active suppression of effector T-cell function.

In addition to changes in T-cell function and increased NKT-cell numbers, NKT-cell cytokine production also appears to change with increasing age. While this area of investigation needs to be explored much further, so far it has been shown that splenocytes activated by CD3ε mAb in vitro show increased IL-4 (both cytokine protein and mRNA) and diminished IL-2 output as compared to young, when measured by ELISA [6, 47]. It has also been demonstrated that CD1d-restricted NKT-cells from aged mice produce lower amounts of IFNγ both at basal levels and stimulation with IL-12 [36, 48]. This apparent NKT-cell dependent change in cytokine profile has been postulated to contribute to the overall decreased immunocompetence in aged animals although it has not been proven. Interestingly, systemic anti-CD1d treatment does not significantly decrease inducible IL-4 production [6] and it was found that in fact, memory T-cell subsets (CD44^{high}CD445RB^{low}NK1.1^{neg}) produce much of the age-associated IL-4 in

response to CD3ε-stimulation, however, they do so in an NKT-cell-dependent fashion [38, 47]. Our laboratory also reported that IL-10 production among spleen cell populations is nearly 10 times higher than the amount produced by splenocyte cultures from young mice. Unlike IL-4, the age-related increase in inducible IL-10 was significantly diminished in splenocytes obtained from aged mice that were given systemic NKT-cell blockade. There is also evidence that NKT-cells from aged mice have impaired IFNγ production, since lower baseline levels and IL-12 induced production of this important cytokine have been noted with aging [36]. Although relatively meager, the current set of data collectively support the notion that as age advances, NKT-cells shift from a more protective, IFN-γ producing phenotype (Th1), to a suppressive (Th2) type phenotype in both mice and humans by increasing IL-4 and IL-10 output.

Lastly, in addition to age-related alterations in cytokine production, NKT-cells from aged mice have also been reported to exhibit decreased cytotoxic capabilities [40, 49]. However, whether this observation applies to all CD1d-restricted NKT-cells throughout the immune compartment vs. other CD1d-unrestricted NKT-cell populations, such as those that exist in the liver, remains to be determined.

6 Summary

In summary, the effects of age on conventional lymphocyte populations have been widely studied, but age-related alterations among innate lymphocytes including NKT-cells are not as well understood. From a thorough review of the current literature that examines elderly humans and truly gerontologic mice, it appears that as age advances, the number of NKT-cells increases and their functions, particularly cytokine production, deviate away from immune protection and more toward immune suppression. The mechanisms responsible for greater numbers of NKT-cells in aged mice is also unclear, but may result from dysregulated ontologic signals that control progenitor cell development and proliferation, cell death, and homeostatic proliferation. Additionally, the possibility exists that NKT-cells, like other cells of the innate immune system may exhibit comparatively greater longevity than conventional lymphocytes. What is clear is that given their potent regulatory capacity over immune and inflammatory processes, significantly more research is required with both human cells as well as mouse models to understand how age-related alterations in NKT-cell biology might contribute to either the age-related decline in immunity or development of cancers and autoimmunity.

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Stem Cells

Lympho-Hematopoietic Stem Cells and Their Aging

Hartmut Geiger and Gary Van Zant

Contents

Abstract: The lympho-hematopoietic system is largely composed of cells with short lifespans (days) and thus requires continuous replenishment of the cells lost through hematopoietic stem and progenitor cells in a process called hematopoiesis. Experimental evidence from several laboratories clearly demonstrates that hematopoietic stem cells (HSCs) harvested from young and aged animals show functional differences that are intrinsic to HSCs, implying that also stem cells in the hematopoietic system can not defy aging. We will thus discuss in this chapter the cellular phenotypes and the possible molecular mechanisms associated with aged HSCs with respect to the specific properties stem cells are endowed with, and will

H. Geiger (✉)
Assistant Professor
Division of Experimental Hematology
TCHRF S7.601
Cincinnati Children's Hospital Medical Center
3333 Burnet Avenue
Cincinnati, OH 45229, USA
Tel.: 513 636 1338
Fax: 513 636 3768
E-mail: hartmut.geiger@cchmc.org

G. V. Zant
Department of Internal Medicine
University of Kentucky, Lexington
Kentucky, USA

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investigate whether stem cell aging is inevitable or whether some of its aspects can be reverted or at least ameliorated.

Keywords: Stem cell • Aging • Hematopoiesis • Niche • DNA damage • Adhesion

1 Stem Cells

Organ or tissue attrition due to loss of cells by various means is inevitably associated with life. Thus to achieve tissue homeostasis for a long period of time, lost cells have to be replaced and/or renewed. Many, but perhaps not all tissues or organs, depend on undifferentiated stem cells to support the generation of novel differentiated cells for a given tissue. Many tissues of the major organ systems are thus composed of short-lived cells that require continuous replenishment like skin, intestine and the hematopoietic tissue as well as somatic stem cells (Potten and Morris, 1988; Morrison et al. 1995; Fuchs and Segre, 2000; Tani et al. 2000; Stappenbeck et al. 2003). Stem cells have been also identified in brain and heart, although their contribution to adult tissue homeostasis is still debated (McKay, 1997; Doetsch et al. 1999; Gage 2000; Beltrami et al. 2003; Oh et al. 2003).

Stem cells are commonly defined by two characteristics: their ability to either self-renew or to differentiate into most of the mature cells types that comprise a tissue (van der Kooy and Weiss 2000). Both processes are associated with the ability of stem cells to undergo symmetric versus asymmetric divisions (mode of the division). The regulation of the mode of division thus poses an important fundamental question in stem cell biology. The molecular determinants that influence symmetric versus asymmetric divisions of stem cells are not well understood, which still hinder rationale approaches to modulate the outcome of stem cell divisions for example for clinical purposes.

2 Hematopoiesis

The lympho-hematopoietic system is largely composed of cells with short lifespans (days) and thus requires continuous replenishment of the cells lost through stem and progenitor cells in a process called hematopoiesis. Hematopoiesis is in adults restricted mostly to the bone marrow (BM) cavities. Hematopoietic stem cells (HSCs) are the most primitive cells of the blood lineage and give, upon differentiation, rise to the entire panoply of mature blood cells. They are rare and comprise only about 0.01% of the BM cell population, but are a long-lived population that are normally not depleted during a lifetime.

The cellular differentiation pathway is organized in a functional hierarchy, in which HSCs differentiate upon an asymmetric division into progenitor cells (also called transient amplifying cells in other stem cell systems), which then differ-

entiate upon multiple steps of additional asymmetric divisions into mature blood cells. Upon differentiation and further specification, hematopoietic progenitor cells (HPCs) lose their ability to self-renew forever, and thus only true HSCs are associated with unrestricted self-renewal capacity.

In addition to their self-renewal and differentiation capacity, HSCs are also endowed with a remarkable, but still not well understood mobilization and homing ability, meaning they are able to migrate out of the BM into the bloodstream and also are able to migrate with a relatively high efficiency and specificity from peripheral blood back into the BM and to their niche, where this self-renewal and differentiation takes place. The physiological role of HSCs found in the circulation has puzzled investigators for a long time. One explanation is that these circulating HSCs are a pool of cells that help distinct sites of hematopoiesis to communicate with each other (Wright et al. 2001). Another interesting recently published explanation is that HSCs apparently circulate into and out of the lymphatic system serving an immuno-surveillance role, and it is suggested that they can via this route survey peripheral organs and foster the local production of tissue-resident innate immune cells under both steady-state conditions and in response to inflammatory signals (Massberg et al. 2007).

Thus, due to these distinct abilities, the potential of hematopoietic stem cells can be tested in a transplantation assay, which is regarded as the gold standard for testing HSCs activity in vivo. In such an assay, syngeneic animals will be myeloablated or lethally irradiated, which opens up in both cases niches for HSCs in the BM. Subsequently, HSCs that are injected into the bloodstream of these recipient mice will home to these empty niches, undergo self-renewal and differentation, and will consequently be able to contribute to the hematopoietic system of the animal for a lifetime. The relative or absolute ability of the transplanted

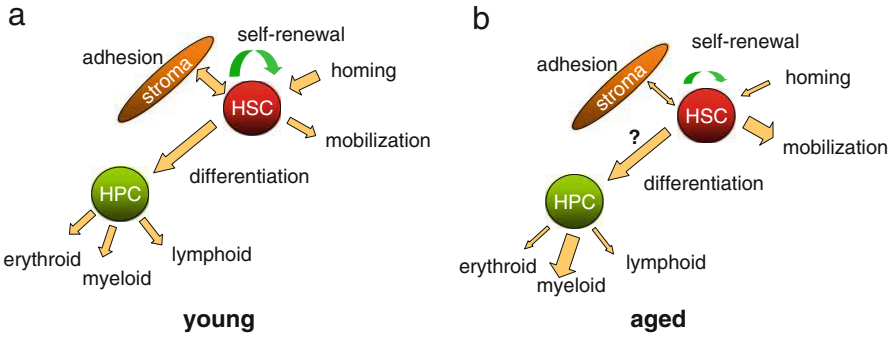

Fig. 1 Aging of hematopoietic stem cells. (a) Hematopoietic tem cells (HSCs) are defined by their ability to self-renew, to home to the bone marrow, to mobilize out of their niche into peripheral blood and their ability to adhere to stroma cells. In addition, HSCs will differentiate via hematopoietic progenitor cells (HPCs) in various distinct blood cell lineages. (b) Upon aging, HSCs present with reduced self-renewal activity, decreased homing but enhanced mobilization ability, which might be a result of the reduced ability of aged HSCs to adhere to stroma. Aged HSCs furthermore show a clear preference for myeloid over lymphoid and erythroid differentiation

stem cells to reconstitute the hematopoietic system of the recipient is regarded as a quantitative measurement of the stem cell potential.

3 How Do We Define and Isolate Hematopoietic Stem Cells?

Under a microscope, hematopoietic stem cell look actually identical to HPCs and similar to even some differentiated hematopoietic cells, including small- to medium-sized lymphocytes. So how do we identify and purify HSCs to study their biology? Over 50 years of intense research in the field of hematopoiesis and HSCs allow researchers now, at least in mice, to prospectively isolate the putative stem cells solely based on the inclusion or exclusion of distinct cell surface markers trough cell sorting via flow cytometry. HSC function and purity can then be subsequently verified in transplantation assays. Various distinct combinations of cell surface markers, which further evolved over time in complexity, but inversely resulted also in higher purity, have been used to identify HSCs. In aggregation of most of the literature, and most widely used by investigators at present, are three protocols to prospectively isolated stem cells, the LSK/CD34, the SLAM and the side population (SP) approach (Osawa et al. 1996; Kiel et al. 2005; Lin and Goodell 2006; Yilmaz et al. 2006). In the LSK/CD34 system, HSCs are defined as being negative for markers found on differentiated cells (lineage markers, and thus LIN- or L) as well as negative for CD34, but positive for the Sca-1 and the c-Kit epitopes. In the SLAM system, HSCs are defined as BM cells positive for CD150 while at the same time negative for CD48. Finally, in the SP approach a distinct cell population that does not retain the Hoechst dye 33342 (measured at two distinct emission wavelength) is highly enriched for HSCs. Although the purity of the presumed stem cell populations varies among these protocols, and each purification system might even enrich for slightly functionally distinct HSCs (Weksberg et al. 2008), all three are rigorously experimentally validated in terms of their ability to highly enrich for stem cells, and of course, there is major cell surface phenotype overlap among the purified populations (Kiel et al. 2005; Yilmaz et al. 2006; Weksberg et al. 2008).

3.1 The Aging of Hematopoietic Stem Cells

The aging process is probably best defined as a general loss in biological competence for both the individual cell and the organism as a whole. At the cellular level, it is expressed as decreasing replicative ability in proliferating cells and decreasing functional activity in postmitotic cells.

Stem cells were thought to be endowed with unlimited self-renewal capacity and thus assumed to be exempt from aging, which would result in functionally young

stem cells in a chronologically aged animal. But evidence accumulating over the past decade has now proven that there is a measurable and successive functional decline in hematopoietic, intestinal and muscle stem cell replicative activity from adulthood to old age, resulting in a decline of stem cell function (Morrison et al. 1996b; Chen et al. 1999; Sudo et al. 2000; Geiger and Van Zant 2002; Kim et al. 2003; Van Zant and Liang 2003; Rossi et al. 2005; Chambers et al. 2007). As stem cell activity is necessary to replenish lost differentiated cells in a stem cell driven tissue, it has been hypothesized that aging of stem cells leads to reduced renewal and thus reduced tissue homeostasis in aged animals, probably most obvious under stress situations (Geiger et al. 2001b; Geiger and Van Zant 2002; Van Zant and Liang 2003; Sharpless and DePinho 2004; Torella et al. 2004).Ultimately, this may determine individual longevity, although so far no lifespan extension in response to stem cell therapy has been reported. This hypothesis though is supported for example by the fact that the function of the innate immune system, which depends on stem and progenitor cells activity, is compromised in aged individuals (Ginaldi et al. 1999; Butcher et al. 2001; Lord et al. 2001).

3.2 Defining Aging of Stem Cells

Stem cells are social entities that communicate and associate with supporting cells (called stroma) in a distinct 3 dimensional space (called niche). It is believed this niche is essential for stem cell regulation (Yin and Li 2006). So immediately the question arises whether stem cells age themselves (intrinsic aging) or the niche itself ages, which as a consequence impairs the function of an otherwise young and healthy stem cell occupying this niche (extrinsic aging). In case stem cell aging was mostly driven by extrinsic clues, we would anticipate that stem cells from aged animals, when transplanted into young niches (young animals), become functionally young again. Experimental evidence though from several laboratories clearly demonstrates that HSCs harvested from young and aged animals show functional differences that are intrinsic to HSCs (Geiger and Van Zant 2002; Geiger et al. 2005; Rossi et al. 2005) and less dependent on the microenvironment, although it does not exclude extrinsic influences on stem cell aging. These data thus allow us to refer to the cells as aged HSCs and young HSCs when the age refers to the animal from which the cells were harvested. Whether or not though stem cell aging in general is mostly driven by intrinsic mechanisms is still a matter of debate, as for example in muscle, aging of muscle progenitor cells is reverted by changes in the systemic environment and thus most likely dominated by extrinsic factors (Conboy et al. 2005).

Various experimental approaches have been developed/employed to study causes and consequences of HSCs aging, and there seems to be a experimental duality in the field of aging research in general and also in the context of HSCs: Research on aging in a physiological environment and at a physiological pace in contrast to research on stem cell phenotypes in genetically modified animals which present

with accelerated aging/stem cell aging. Let us have a short detour to the car repair shop to explain the differences in more detail.

We will start with describing the ailments of old cars (old stem cells) and just list all the problems a mechanic usually finds in them and which might be the reason for why the old car might not function very well anymore. We know that the mechanic's observations are correlative, but he will eventually replace/fix the worn out part(s) he was talking about and thus will subsequently test whether he was guessing right (causative approach).

Genetically modified animals on the other hand that present with phenotypes indicative of accelerated stem cell aging (either partial or in general) are more like cars in which we messed up on purpose with let's say the water pump and of course, over time the pump and subsequently the car will break. This tells us that the water pump is an essential part for the car, it does though not prove that in most cases in aged cars that is the part that will break, or whether preventing in general water pumps from braking in old cars will reduce the fragility of old cars. Only after the mechanic that usually sees all the old cars in the repair shop agrees that water pumps in old cars are an issue and tend to break, we are convinced about the relevance of our observations in the accelerated model [*see* also (Hasty and Vijg 2004; Miller 2004; Warner 2004)]. Although the authors do not imply that one approach might be superior over the other, it is still a good idea to differentiate between these experimental approaches. We will focus in the following paragraph primarily on physiological stem cell aging, and refer to premature aging systems whenever relevant.

There is still controversy on aspects of the phenotype and function of physiologically aged HSCs. The following phenotypes therefore represent commonly agreed phenotypes for aged HSCs and supported by research conducted by multiple independent investigators (canonical aging phenotypes). We suggest that the combination of these phenotypes comprise an physiologically aged HSCs, but that of course "partial" stem cell aging might be observed under given circumstances. Aged and young HSCs differ mostly in their function, and for HSCs, this function is best measured in transplantation assays. Consequently, most of the available data characterizing functional differences between aged and young stem cells has been generated by comparing young and aged HSCs in transplantation experiments.

3.3 Changes in Function Associated With Aged HSCs

In a competitive transplant setting, when stem cells from aged mice from the C57BL/6 inbred strain are transplanted along-side young cells into a lethally irradiated recipient animal, aged HSCs are less efficient in contributing to hematopoiesis (perturbed homeostasis) compared to young HSCs (Morrison et al. 1996b; Chen et al. 2000). Whether or not aged HSCs are also reduced in their ability to self-renew is still a matter of debate, although clearly aged HSCs are functionally impaired compared to young stem cells in serial transplantation assays.

Aging also affects the differentiation potential of HSCs (Rossi et al. 2005). Many studies have demonstrated that aged stem cells have a reduced ability to support the red blood cell system, and that aged HSCs do not efficiently generate both T- and B lymphoid progeny, while they present with an increased ability to differentiate into the myeloid lineage [*see* (Linton and Dorshkind 2004) and references cited therein]. This difference in cell lineage self-renewal is emphasized by age-associated anemia and a decline in function of immune cells in aged individuals (Lipschitz and Udupa 1986; Lipschitz 1995; Ginaldi et al. 1999; Sudo et al. 2000; Butcher et al. 2001; Lord et al. 2001; Kim et al. 2003; Rossi et al. 2005). The generalized lymphoid defect has been at least in part attributed to an impaired ability of aged HSCs to differentiate into the common-lymphoid progenitor cell, the progenitor cells that will give rise to both the T- and the B-cell lineage. Thus aged HSCs are impaired in their ability to support the repopulation of the thymus and are less able to contribute to the B-cell as well as the T-cell lineage. Finally, aged HSCs are reduced in their ability to home from PB into the BM upon intravenous injection. A reduced ability to home to BM of aged HSCs was implied/speculated about in various publication and was recently experimentally confirmed by Liang et al. (Sudo et al. 2000; Kim et al. 2003; Liang et al. 2005; Rossi et al. 2005). The ability of HSCs to home to BM out of PB is clinically very important, as this is the first step to achieve successful engraftment in a HSC transplantation setting. Interestingly though, aged HSCs show enhanced mobilization proficiency upon G-CSF (Xing et al. 2006), which might in combination with the reduced homing indicate reduced cell–cell adhesion parameters for aged HSCs. Both impaired homing by old HSC and enhanced mobilization might imply "looser" niche association including dysregulated proteins involved in the HSC–niche interaction.

Comparing the engraftment properties of HSCs from various aged mouse inbred strains revealed that there are considerable differences in the rate of which stem cell self-renewal activity is reduced in aged animals, suggesting a strong genetic regulation of stem cell aging (Van Zant et al. 1990; Chen et al. 2000; Chen 2004; Geiger et al. 2005; Snoeck 2005;). Old C57BL/6 animals for example present with an increase in the number of phenotypically defined stem cells, although each of these stem cells has a clearly reduced potential upon transplantation, whereas old DBA/2 animals present with both, a reduced phenotypic number and a reduced function for aged HSCs. Thus, although overall the potential of the stem cell population decreases in the BM with aging independent of the strain, the pace with which this happens seem to be genetically restricted, adding another level of complexity to the determination of mechanisms that result in stem cell aging.

While the determination of stem cell function in the murine system via the transplantation assay can be relatively easily accomplished, our experimental tools to determine the function of human stem cells are understandably more limited. Thus investigations into the function of aged human HSCs are still in their infancy and more open to speculation, although both a reduced ability to support T-cell development as well as a reduced clonality and thus reduced stem cell number in aged humans has been so far identified [*see* for example (Wick et al. 1989; Abkowitz et al. 1998; Offner et al. 1999)]. In addition, elderly patients (similar to mice) fre-

quently present with anemia. The mechanisms for this finding are mostly unexplained. (Balducci et al. 2006; Ferrucci et al. 2007).

3.4 Molecular Phenotypes Associated With Aged HSCs

What might be the molecular mechanisms responsible for the age-associated decrease in HSC function? As obvious from the previous paragraph, there are multiple phenotypes associated with aged stem cells. We thus do not assume that there is a single, unique molecular mechanism of stem cell aging, but rather that there are most likely multiple molecular pathways that result in these phenotypes, which we will address below.

3.5 Genome-Wide Gene Expression Profiling of Aged HSCs: Finding Pathways in Aging?

Several laboratories recently undertook a global approach to identify on the genome scale changes in the transcription level of genes associated with the young/aged transition of HSCs (Chambers et al. 2007; Rossi et al. 2005, Geiger and Van Zant, unpublished results). Unfortunately, each of these experiments were performed with HSCs purified according to a different scheme and analyzed with distinct microarrays, rendering a comparative approach almost impossible. In general though, genes associated with the stress response, cell adhesion, protein turnover and signal transduction dominated the up-regulated expression profile, while the down-regulated profile was marked by genes involved in the preservation of cell adhesion, genomic integrity and chromatin remodeling. So far though no specific pathway based on these RNA expression analyses could be identified based on the collections of these differentially expressed genes, emphasizing probably one more time the complexity of aging even at the single cell level. Interestingly though, in one set of these experiments, gene products associated with myeloid leukemia were markedly upregulated, which the authors interpreted in the way that aged stem cells might be, through their altered expression profile, already intrinsically prone to leukemia, although this hypothesis will require further experimental testing (Rossi et al. 2005).

3.6 The Role of Oxidative Damage in Stem Cell Aging

An important and probably universal mechanism that leads to a wide spectrum of intracellular damage during aging is extended exposure to reactive oxygen species (Hasty 2001). Long-term exposure to these metabolic byproducts leads to structural changes in a number of cellular macromolecules that impair their function. Such changes include the cross-linking of intracellular and intra-mitochondrial structural

and functional proteins and carbohydrates, and the oxidation of fats and lipids in membranes as well as DNA damage. The multiple functional components a cell consists of form complex and interdependent physiological systems, making it difficult to determine which age-related change may be the primary cause of aging and which changes may be entrained by the primary event. Age-dependent changes in mitochondrial function and DNA integrity due to the accumulation of respiratory oxidative stress have also been reported for a variety of cell types, including liver, intestinal crypt and cardiac muscle cells (Bohr et al. 1998; Taylor et al. 2003). But the importance and frequency of mitochondrial mutations might have been overestimated compared with somatic mutations, as no increase in mitochondrial mutations in normally aged mice could be detected (Anson et al. 2000; Jacobs 2003; Khrapko and Vijg 2007; Vermulst et al. 2007). A role for reactive oxygen species and the p38 MAPK activity in limiting the self-renewal capacity and thus the lifespan of HSCs was recently experimentally demonstrated, as antioxidative treatment of HSCs resulted at least in partial reversion of the phenotypes associated with aged stem cells (Ito et al. 2006).

4 The Role of Telomere Length/Telomerase in Aging of HSCs

The hypothesis that cellular senescence is mediated via replicative exhaustion (Hayflick and Moorhead 1961; Barker et al. 1982) has received mechanistic support from the finding that telomeres may act as a mitotic clock (Vaziri et al. 1994; Greider 1998). Telomeres, the repetitive sequences at the chromosomal ends that provide chromosome stability, shorten with each round of cellular replication. When a critical short telomere length is reached, the cell enters a state of senescence or apoptoses. Some reports suggest a small, but significant decrease in telomere length in either human or murine HSCs upon aging or replicative stress post transplantation [(Vaziri et al. 1994; Brummendorf et al. 2001; Lansdorp 2008), and unpublished data Van Zant]. Since HSCs from laboratory mouse strains present with telomeres several times longer than human cells (Hemann and Greider 2000), and since stem cells synthesize telomerase to maintain telomeric length (Morrison et al. 1996a), whether natural telomere shortening by itself plays a role in HSC aging is still a matter of debate. These observations are further supported by the finding that although loss of telomerase activity clearly results in phenotypes associated with premature aging of stem cells, over-expression of telomerase in HSCs could so far not revert stem cell aging (Allsopp et al. 2003a, b).

5 The Role of DNA Damage in Aging of HSCs

A more likely cause for aging of stem cells in general, and HSCs in particular, might be age-dependent acquisition of defects in genomic DNA. HSC or intestinal stem cells show a high radiation sensitivity compared with most other cell

types (Jacobson et al. 1949; Martin et al. 1998). This radio-sensitivity implies either a reduced ability of stem cells to repair even small amounts of DNA damage, stringent requirements on the DNA repair machinery for the maintenance of DNA fidelity, or increased rates of apoptosis. These various possibilities are not mutually exclusive. Moreover, brain, liver and muscle cells from old mice have a reduced ability to repair radiation-induced damage compared to young animals (Hamilton et al. 2001). Furthermore, Dolle et al. reported a general increase in the frequency of genomic mutations in old compared with young animals. Interestingly, small intestine, the only stem cell system with a high cell turnover analyzed in these experiments, showed the highest mutation frequency in 2.5-year-old animals among other tissues like heart, brain and liver (Vijg and Dolle 2002), although recent results indicated that the majority of this increase is attributed to a specific cell type in the organ/tissue, and thus does not affect all cells (Busuttil et al. 2007). Loss of DNA integrity with age as the major cause of stem cell aging is also compatible with the finding that aging of HSCs is mostly cell autonomous (van der Loo and Ploemacher 1995; Geiger et al. 2001a; Rossi et al. 2005; Rossi et al. 2007). Research to determine DNA-repair capacity in mammalian systems has mostly been concentrated on the quantification of DNA damage in whole tissues in response to induced damage by utilizing cell culture or animal models (Gaubatz and Tan 1994; Jeng et al. 1999; Goukassian et al. 2000; Doria and Frasca 2001; Zhao and Hemminki 2002; Beausejour et al. 2003; Chevanne et al. 2003; Parrinello et al. 2003; Scarpaci et al. 2003;). Although published results are contradictory, the majority of the data supports the notion that the DNA repair capacity declines with age and that aged HSC present with elevated levels of DNA damage (Mullaart et al. 1990; Rossi et al. 2007). HSC, similar to other types of stem cells, show an increased radiation sensitivity compared to mature cell types (Jacobson et al. 1949; Martin et al. 1998) and a distinct expression pattern of DNA-repair genes (Geiger lab, unpublished). Both facts imply that stem cells might use DNA repair pathways differently compared to well-studied pathways in differentiated cells or cell lines, a hypothesis supported by research on DNA repair pathways in ES cells (Cervantes et al. 2002; Hong and Stambrook 2004). In addition, the expression of the cyclin-dependent kinase inhibitor p16INK4a, a stress/DNA damage indicator, was found to be elevated in physiologically aged HSCs and this has been shown to be causative for reduced survival of aged HSCs under stress, as loss of p16 expression ameliorated stem cells aging (Janzen et al. 2006). Taken together, changes in the DNA repair system in HSCs, together with changes in cell cycle regulation due to DNA damage with age, might be an important cause for the decrease in the functional capacity of aged HSCs or in general aging of multiple tissue. Such a connection is further supported by comparative linkage analysis of hematopoietic stem cell traits and longevity (Geiger and Van Zant 2002). Loci mapped to chromosomes 2, 7 and 11 regulate DNA repair and aging of primitive hematopoietic cells and at the same time, longevity (Geiger et al. 2001a). As so far though only partial amelioration of HSCs aging could be achieved by altering expression of DNA repair genes or by antioxidative therapy,

whether the DNA damage response plays the major role in stem cell aging will still be a matter of debate.

6 Altered Stem Cell–Niche Interactions in HSC Aging: A Novel Player in the Game

HSCs are entities that have social interactions. They reside in specialized three-dimensional microenvironments, or niches, in the BM. Cell-cell adhesion interactions between HSCs and stroma cells in the niche are believed to regulate HSC proliferation and differentiation. So far two niches, an endosteal and an endothelial niche, have been identified in the BM, and the distinct contribution of both of them to hematopoiesis is currently discussed [*see* for example (Adams and Scadden 2006; Kiel and Morrison 2006; Scadden 2006; Wilson and Trumpp 2006)]. Interactions of HSCs with stroma cells in the niche are consequently considered to be central to the biology of HSCs and have been therefore referred to as a stem cell synapses (similar to the immunological synapse; Adams and Scadden 2006; Scadden 2006; Yin and Li 2006). We recently reported that aged HSCs are impaired in their ability to strongly adhere to stroma cells [unpublished results and (Xing et al. 2006)]. This observation is supported by the fact that for example, the integrins $\alpha 4$ and $\alpha 5$ and the cell adhesion molecule VCAM-1 show lower expression on aged HSCs, whereas the expression of the adhesion molecule P-selectin and the $\alpha 6$ integrin are elevated in aged HSCs (Rossi et al. 2005; Xing et al. 2006). We could further demonstrate that physiologically aged primitive hematopoietic cells presented with elevated activity of the small Rho-GTPase CDC42, a protein tightly involved in regulating cellular adhesion (Van Hennik and Hordijk 2005; Wang et al. 2007; Yang et al. 2007). A distinct role for altered expression of adhesion molecules and thus altered adhesion in HSC differentiation was recently also suggested by Forsberg et al. (Forsberg et al. 2005), which supports a model in which unstable adhesion of aged HSCs to stroma might be causative for subsequent functional changes in aged HSCs. Whether the correlation between changes in expression of adhesion receptors and changes in the function of aged HSCs is also of mechanistic relevance though is not clear at the moment. We subsequently proposed that aged primitive hematopoietic cells are impaired in their ability to interact efficiently with stroma cells, which might result in the reduced self-renewal capacity as well as the altered differentiation ability associated with aged HSCs (Geiger et al. 2007). Elevated level of CDC42 activity detected in aged hematopoietic cells might be causative for these changes in cell–cell adhesion. These findings would further imply that also the stroma might play an important role in stem cell aging, as it too might be reduced in its ability to strongly interact with HSCs when aged. Molecular mechanisms that result in altered adhesion dynamics and altered function of aged HSCs might thus be tightly interconnected and result in an altered stem cell synapse for aged HSC. But as so many hypotheses linked to aging of stem cells, also this one is in critical need of additional experimental validation.

7 Amelioration/Prevention of Stem Cell Aging

Stem cell aging might be the underlying cause for dysregulated tissue homeostasis in aged individuals and consequently attenuation of stem cell aging will become a central to regenerative medicine. Identifying conditions under which in an aged organism aged stem cells are activated to be functionally equivalent to young stem cells could thus be a first step towards designing treatments to attenuate/revert the consequences of stem cell aging and consequently to improve age-associated imbalances in tissue homeostasis.

Studying muscle regeneration by muscle stem cells (satellite cells) in aged animals, Conboy et al. recently reported that aged muscle stem cells can be activated to repair/differentiate as efficiently as young muscle stem cells either by forced activation of Notch, or by systemic factors provided by serum from young animals (Conboy et al. 2003, 2005). They also identified that factors in serum from aged mice negatively affect muscle stem cell activation. As aging of HSCs is at least in part cell intrinsic, amelioration of HSCs aging might be more difficult to achieve. As mentioned above, loss of the p16 protein as well as antioxidative therapy both resulted in partial reversion of HSC aging. In addition, attenuation of HSC aging was also achieved by lifelong caloric restriction of mice from the BalbC inbred strain, without though further identification of a possible molecular mechanism underlying this stem cell response (Chen et al. 2003). These results are very promising and prove that it is possible to change the path of stem cell aging. More research though will be necessary to translate our knowledge on stem cell aging into therapies that promise a lifelong fountain of stem cell youth to all of us.

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Implications of Developmental Switches for Hematopoietic Stem Cell Aging

Jens M. Nygren and David Bryder

Contents

Abstract: Each of the different hematopoietic cell types has their own properties and function, but only when they all act in tight synergy are they able to constitute a highly specific and efficient immune defense capable of efficient protection from invading pathogens and appropriate maintenance of blood clotting and oxygen transport functions.

All blood cell types are continuously produced in the bone-marrow by rare hematopoietic stem cells that persist throughout the life of the organism. These stem cells are influenced by their environment and developmental history and experience a range of cell intrinsic changes that over time alter their functional properties. These timed changes include alterations in fundamental processes such as self-renewal, proliferation, differentiation and gene expression, thereby being crucial for both normal maturation as well as hematopoietic aging.

Keywords: Aging • Developmental switch • Hematopoietic stem cell • Ontogeny • Quiescence

D. Bryder · J. M. Nygren (✉)
Stem Cell Aging, Department of Experimental Medical Science,
BMC D14, Lund University, 221 84 Lund, Sweden
Phone: +46 (0)46 22 20 313
Fax: +46 (0)46 22 24 218
E-mail: jens.nygren@med.lu.se

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1 Introduction

The blood system produce hundred of millions of new blood cells everyday to maintain oxygen transport, blood clotting and immune function [Morrison et al. 1995b]. This process is highly conserved through evolution and therefore largely similar between lower vertebrates and mammals [Laird et al. 2000; Zon 1995].

In the early 1960s, McCulloch and Till performed a series of ground breaking experiments to search for multipotent and self-renewing hematopoietic stem cells (HSC) that could sustain such extensive blood cell production throughout life. Bone-marrow cells were injected intravenously into irradiated mice and the subsequent engraftment of a rare fraction of these cells, as visible nodules in the spleens of the recipients, were evaluated. From such experiments, it was established that nodules appeared in proportion to the number of bone-marrow cells injected, and that individual nodules arose from single bone-marrow cells, named colony forming units spleen (CFU-S) [McCulloch and Till 1960]. The ability of CFU-S to self-renew [Becker et al. 1963], a cardinal property of stem cell function, provided the first true evidence that somatic HSC exists in the bone-marrow. Today, four decades later, we know that the bone-marrow is the primary hematopoietic organ by the end of fetal maturation and that seeding of HSC to the bone-marrow is a uniform process and results in homogeneous distribution to the different bone-marrow compartments, without spatial differences in hematopoiesis or HSC identity [Kiel et al. 2005]. The majority of cells in the bone-marrow are maturing blood cells and their progenitors, and thus HSC constitute a rare population of less than one in 15,000 bone-marrow cells [Lagasse et al. 2001].

2 Quiescent Hematopoietic Stem Cells

The identity of the bone-marrow HSC cannot be recognized by their morphology and phenotype alone, but rather by their unique functional properties [Lagasse et al. 2001; Matsuzaki et al. 2004; Osawa et al. 1996]. Traditionally, function is evaluated by the ability of cells to reconstitute the blood system following transplantation into hosts in which endogenous HSC have been eradicated by a lethal dose of irradiation. Similar to the CFU-S assay, reconstituting clones can be assayed by tissue sampling. The progeny from reconstituting HSC are identified by monoclonal antibodies and detected by flow cytometry to establish both level and quality of engraftment. With this assay, HSC properties can be analyzed even at a clonal level by detection of the descendants from single transplanted HSC [Osawa et al. 1996]. However, although ultimately being capable of producing all blood cells, including phenocopies of themselves, HSC remain low proliferative, presumably to limit divisional stress and any intrinsic changes that comes with accumulated cell divisions and aging. The mechanisms underlying such regulated quiescence is currently unknown but is one of many features that take place throughout development which eventually lead to the functional changes that are ascribed to the aging of HSC.

2.1 Quiescence and Cell Cycle Control

Adult HSC are largely quiescent in that their transit through the cell cycle is slow or even arrested at times. This is reflected by that as few as eight percent of the cells in the HSC pool enter cell cycle every day. Nonetheless, quiescence does not result in cell cycle arrest as most cells within a population of HSC have divided at least once within four to eight weeks [Bradford et al. 1997; Cheshier et al. 1999]. The series of events in an eukaryotic cell that are referred to as the cell cycle consists of distinct phases in which the cell undertakes sequential actions like growth and preparation of the chromosomes for replication (G_1 phase), DNA synthesis to duplicate the chromosomes (S phase), additional growth and preparation for cell division (G_2 phase) and finally, mitosis (M phase) during which the cell divides into 2 daughter cells [Steinman 2002]. This cyclic process is regulated at checkpoints [Mantel et al. 2001; Pardee 1989; Steinman 2002] during each phase-transition by cyclins that form complexes with cyclin-dependent kinases [Cheng 2004; Steinman 2002]. The cyclin-based surveillance system acts as a quality control that monitors the cell as it progresses through the cell cycle. Checkpoints can block progression through one phase if certain conditions are not met. For instance, mitosis is inhibited until DNA replication is completed or if not all chromosomes are attached to the mitotic spindle. A surveillance network of signaling molecules has been set up to instruct cells to stop dividing and to either repair the damage or initiate programmed cell death if necessary. For cells, like HSC, that persists and continues to proliferate throughout life, quiescence through tight negative control of cell cycle propagation and stringent surveillance of DNA integrity appears essential to minimize divisional stress and to ensure that damaged cells are not further propagated and do not progress into a cancerous state.

Some cells leave the cell cycle at the G_1 phase following a cell division and enter a nonproliferative G_0 stage [Pardee 1989]. Most often, G_0 cells are terminally differentiated and their exit from the cell cycle is thus permanent, whereas other cells, like HSC, are only temporally quiescent and can upon mitotic stimulation re-enter G_1 and prepare for additional cell cycles [Bradford et al. 1997]. HSC are relatively unresponsive to mitogenic stimuli [Bradford et al. 1997; Huang et al. 1999; Uchida et al. 2003] and this might reflect the fact that most cells in the HSC population are in a G_0 state. Thus, that HSC need a longer time and stronger stimulation than committed progenitor cells to respond to growth factors might reflect that they first need to get activated in order to reenter the cell cycle. Distinct regulation of the cell cycle activity of HSC by factors known to limit proliferation and differentiation [Cheng et al. 2000; Hock et al. 2004; Iwama et al. 2004; Lessard and Sauvageau 2003; Park et al. 2003; Walkley et al. 2005] is therefore presumably a key requirement to avoid exhaustion of the HSC compartment [Iscove and Nawa 1997] and should therefore represent a defining stem cell property. Active cell cycling have been suggested to exert negative effects on stem cell function [Fleming et al. 1993a; Glimm et al. 2000; Habibian et al. 1998; Jetmore et al. 2002; Orschell-Traycoff et al. 2000] although this can at least in part be an interpretation of experimental observations,

as the design of such experiments have assumed both that dividing hematopoietic stem and progenitor cells have a similar cell cycle transit time and that HSC are identifiable by phenotype alone. Most likely, HSC in cycle are functionally normal but due to their quiescent state represents a rare fraction within populations of enriched HSC in S/G_2/M stages. They are therefore outnumbered by nonquiescent and transiently reconstituting multipotent and lineage committed progenitor cells, which dominate most HSC enriched populations established to date [Nygren et al. 2006].

2.2 Quiescence but yet Hematopoiesis

As HSC divide, they can produce daughter cells of which at least one represent an identical replica of its ancestral HSC. Such self-renewing cell divisions are a hallmark of stem cells and necessary to maintain a constant HSC pool and lifelong production of all blood cell types [Becker et al. 1963]. Maintenance of the HSC pool can be the result of either asymmetrical cell divisions [Jan and Jan 1998] that results in one cell that is identical to the mother cell and one cell that is committed to differentiation, or of a balance of symmetrical cell divisions leading to either complete self-renewal or differentiation (i.e., result in either 2 HSC or 2 committed daughter cells). Nevertheless, asymmetric HSC divisions must occur at some point during cellular development and multilineage differentiation of committed cells [Takano et al. 2004] in order to appropriately generate progeny both for daily blood cell production as well as maintenance of a fairly constant number of slowly proliferating and inactive HSC. Daughter cells that do not inherit a stem cell identity loose the regulatory circuitry that limits proliferation by inhibiting mitogenic stimulation, and leave the quiescent state. Proliferation at the stages beneath the stem cells is likely to be an important regulator of differentiation as hematopoietic maturation requires signaling from the cyclin based surveillance system for proper influence on differentiation decisions [Ezoe et al. 2004].

The mechanisms that underlie and direct the multilineage commitment processes from HSC are still largely unknown, but several descriptive propositions of the differentiation processes has been established [Adolfsson et al. 2005; Katsura 2002; Kondo et al. 2001; Yang et al. 2004]. Common for these proposals is that self-renewing and multipotent long-term HSC exist throughout life as they throughout development maintain a quiescent state relative other hematopoietic cells [Nygren et al. 2006]. Their committed progeny irreversibly transit into short-term HSC that are also multipotent, but contribute to hematopoiesis for less than six to eight weeks, as such cells have lost the ability to extensively self-renew [Adolfsson et al. 2001; Yang et al. 2004]. Such transiently reconstituting HSC thereafter commit and enter progenitor states with restricted lineage potentials, to finally develop along certain cell lineage pathways with sequential restrictions in lineage potential and gene expression [Adolfsson et al. 2005; Akashi et al. 2003]. In light of the relatively short life span of a mouse (~2–3 years depending on strain), the low cell cycle

activity among HSC, with a population turnover of several months [Cheshier et al. 1999], is remarkable. If the steady state is severely disrupted, for example following manipulative treatments such as myeloablation and bone-marrow transplantation, HSC react by rapid expansion in the recipient [Allsopp et al. 2001; Iscove and Nawa 1997; Plett et al. 2002] and under the influence of exogenous growth factors, commitment, migration or even self-renewal might occur [Bodine et al. 1993; Fleming et al. 1993b; Kronenwett et al. 2000]. This argues for that the activity of the HSC needs tight negative and intrinsic regulation to fulfill the requirements in different physiological conditions and for the entire life of the organism.

2.3 Quiescence Imposed by a Stem Cell Niche

It is currently unknown whether there is a default fate regarding aspects such as self-renewal or differentiation into individual lineages from HSC. Whereas coordinated and precise control of commitment and differentiation largely depends on soluble factors, originating from within and outside of the bone-marrow, maintenance of HSC identities is believed to be mainly achieved by delicate interactions of the stem cells and their microenvironment, often referred to as the HSC niche. Identification of the factors and their signaling pathways underlying such control has been a main focus of hematological research, as knowledge on such regulation would allow manipulation for therapeutic purposes.

Extrinsic factors produced either by the stem cells themselves or by surrounding stromal cells that bind to receptors on the cell membrane of the stem cell can exert their effects long range as soluble molecules, or locally through direct cell-to-cell contact between the stem cells and adjacent cells [Attar and Scadden 2004]. Stromal cells and their products are spatially distributed into niches that differ in their HSC maintenance capacity and therefore, homing of HSC and hematopoietic progenitor cells to different niches affects their fate and the regulation of hematopoiesis [Arai et al. 2004; Calvi et al. 2003; Zhang et al. 2003]. Under physiological conditions, but more pronounced during stress, HSC migrate in and out of the bone-marrow compartment [Dorie et al. 1979]. This occurs at a very low frequency [Abkowitz et al. 2003; Dorie et al. 1979; Wright et al. 2001] by a mechanism that can be stimulated by exogenous cytokine treatment [Kronenwett et al. 2000]. The purpose and regulation of this migratory activity is not completely understood but might play a role in the seeding of HSC to other niches within the bone-marrow [Abkowitz et al. 2003; Wright et al. 2001], secondary lymphoid organs, like the thymus [Schwarz and Bhandoola 2004] or other organs [McKinney-Freeman and Goodell 2004].

The best direct evidence for stem cell niches comes from work in the Drosophila testis, where germline stem cells surround apical hub cells at the tip of the testis, which provide self-renewing signals [Kiger et al. 2001; Tulina and Matunis 2001]. As the stem cells divide, the daughter cell that keeps contact with the hub cells, and thereby continues to receive self-renewing signals, retains a stem cell identity. The other daughter cell that is relocated away from the hub cells initiates differentiation.

In the bone-marrow, similar regional patterning of self-renewing signals has been found within the endosteal zone lining the bone surface in the marrow cavity [Arai et al. 2004; Calvi et al. 2003; Zhang et al. 2003], with a gradient decreasing toward the central zone of the marrow space. In support of this, the majority of hematopoietic stem and progenitor cells has long since been known to be distributed preferentially along the bone surface [Lambertsen and Weiss 1984; Lord and Hendry 1972; Lord et al. 1975]. These findings suggest that fate determination of HSC within the endosteal zone occurs in a similar fashion as for germline stem cells in Drosophila testes, where the fate of the 2 daughter cells from a dividing HSC at least in part is determined by their attachment to or displacement from the stem cell-supportive niche [Wilson and Trumpp 2006].

2.4 Regulating Quiescence or Commitment

Asymmetry of the daughter cells derived from HSC self-renewing divisions might be due to asymmetric distribution of intrinsic factors, such as transcription factors, cellular components or DNA during cell division [Enver et al. 1998; Takano et al. 2004]. However, HSC regulation is a complex process involving both intrinsic and extrinsic factors that can be both counteracting and synergistic and hence, asymmetric cell division through asymmetric distribution of intrinsic factors might be dependent on extrinsic signals that prime HSC for the subsequent intrinsic regulation. This might explain why extensive efforts to ex vivo expand HSC so far has, with some exceptions, been fruitless [Bryder and Jacobsen 2000; Glimm and Eaves 1999; Miller and Eaves 1997; Moore et al. 1997; Quesenberry et al. 2002; Sauvageau et al. 2004; Srour et al. 1999]. Regardless of the outcome from an asymmetric HSC division, the interaction of the 2 daughter cells with their environment results in fate decisions that determine the destiny of each particular cell and such interaction continues throughout the life of the cell. The newly formed cells can either return to a quiescent state as the parental HSC, carry on a second self-renewing asymmetric (or symmetric expanding) cell division, commit to differentiate along a certain lineage pathway or migrate to a distant site that offers suitable environment for either of the fates above [Wagers et al. 2002]. If the environment does not support any of these possibilities, the cell will due to the lack of instructive signals inevitably undergo apoptosis, a process that plays an important regulatory function for hematopoietic homeostasis [Domen and Weissman 2000; Wagers et al. 2002].

Regulation of these HSC fate decisions is most likely a combination of stochastic (random) events, mainly though intrinsic regulation at the time of cell division [Enver et al. 1998; Greaves et al. 2003; Ogawa 1999; Phillips et al. 1992; Till et al. 1964], and deterministic events, mainly due to extrinsic factors in the HSC niche, that can be either permissive or instructive in their action [Metcalf 1998; Morrison and Weissman 1994; Muller-Sieburg et al. 2002]. Eliminating single or multiple hematopoietic growth factors or signal transduction pathways by genetic engineering in mice, allows determination of the type of action a factor imposes on the

HSC. The action of some factors is redundant as their removal does not result in hematopoietic phenotypes and can be compensated for [Akashi et al. 1998; Metcalf 1993; Sitnicka et al. 2002] whereas others are indispensable for certain fates [Iwasaki et al. 2005]. The first case exemplifies permissive regulation, allowing cells to differentiate along a predestined differentiation pathway, and the latter instructive regulation, instructing cells toward a specific fate [Kondo et al. 2000].

A major group of extrinsic factors in hematopoiesis are cytokines that play an important role in regulation of hematopoiesis. Most such molecules are available both in soluble and cell membrane bound forms and interact by direct binding to cell membrane receptors on the hematopoietic cells or on intermediate cells with which the hematopoietic cells interact. The early acting cytokines Stem Cell Factor and Thrombopoietin are nonredundant regulators of the HCS-pool [Zhu and Emerson 2002]. Other cytokines act on more committed cells and drive differentiation along particular pathways, like Erythropoietin for erythroid cells, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) for myeloid cells and interleukin 7 (IL-7) for B-cells [Ogawa 1993; Zhu and Emerson 2002]. Signaling from membrane bound receptors on HSC is propagated through elaborate intracellular signal transduction pathways to the nucleus, where they influence the activity of transcription factors. These bind to promoter elements on the DNA and regulate together with other regulatory molecules and DNA polymerases the transcription of target genes and ultimately, fate decisions. Several transcription factors have been implicated in the regulation of HSC self-renewal (ICN/CSL, Ikaros, HoxB4 and GATA-2), whereas others participate at more downstream cellular levels by inducing commitment toward individual hematopoietic lineages [Zhu and Emerson 2002].

3 Hematopoietic Stem Cell Developmental Switches

Development of a fully functional blood system occurs early in development and the hematopoietic system thereafter continuously changes to meet the demands on the organism at each stage of development. These changes mainly occur through alterations of identities and functions of the HSC through specific and seemingly irreversible switches that are mainly cell autonomous but depend on and are influenced by variation of the cellular environment of the HSC niches throughout ontogeny.

3.1 The Primitive to Definitive Switch

Onset of hematopoiesis is an early event in embryonic development and required to meet the demands of oxygen transportation as the embryo becomes larger and to provide an early defense against pathogens. In mouse development, gastrulation starts 7.5 days post conception (dpc) and leads to the formation of ectoderm, mesoderm

and endoderm. During this process the extra- and intra-embryonic regions, the yolk sac and embryo proper, are established. In the yolk sac, cell aggregates called blood islands are formed and contain cells of both hematopoietic and endothelial lineages. These develop in close contact, and perhaps from a common progenitor cell known as the hemangioblast [Choi et al. 1998; Keller et al. 1999; Mikkola et al. 2003]. Commitment toward a hematopoietic fate occurs through the influence of various transcription factors, such as Tal-1/SCL, AML-1, Lmo2 and GATA-2, and results in the formation of committed primitive hematopoietic precursor cells [Zhu and Emerson 2002]. Such primitive precursors are primed towards myelo-erythroid lineages and mostly produce monocytes, for infectious defense in the placenta, and primitive erythrocytes, that are large, nucleated and produce embryonic globins [Weissman 2000]. This early burst of extra-embryonic erythrocyte production is necessary for oxygen transportation within the embryo at a time when oxygen diffusion from maternal circulation becomes insufficient [Cumano and Godin 2001]. The yolk sac remains a hematopoietic organ until the embryo itself can support blood cell production by around 11.5 dpc. Within the embryo, a region comprising the rudiments of the dorsal aorta and surrounding splanchnic mesoderm forms at around 8.5–10 dpc and at this site the development of definitive HSC initiates [Bertrand et al. 2005; de Bruijn et al. 2002; Godin et al. 1999]. The region, named the para-aortic splanchnopleura (P-Sp), later develops into the aorta gonad and mesonephros (AGM) at 10–12 dpc. Hematopoietic precursor cell activity can be identified by 10.5 dpc in a region of the mesenchyme surrounding and within the dorsal side of the aorta [Bertrand et al. 2005; de Bruijn et al. 2002; Godin et al. 1999]. These precursors develop into the HSC that support definitive hematopoiesis yielding small enucleated erythrocytes that express adult globins. The intra-embryonic definitive HSC are the sole precursors of the adult HSC that supply HSC activity throughout life [Cumano and Godin 2001], thus no further HSC are generated during late fetal and neonatal stages of development [Gothert et al. 2005]. The origin of the intra-embryonic definitive HSC has been under debate as to whether they really are descendants from the primitive yolk sac derived precursors or originate independently from definitive hematopoietic precursors. Studies by Moore and Metcalf in 1970 suggested that the yolk sac is required for both primitive and definitive hematopoiesis in mice [Moore and Metcalf 1970]. Culture of precirculation 7 dpc embryos from which the yolk sac had been removed developed without blood cell formation, whereas culture of 7 dpc yolk sac alone yielded abundant hematopoietic colonies. Although challenged over the years [Cumano et al. 2001; Medvinsky and Dzierzak 1996], this concept was recently confirmed by noninvasive labeling of progenitors of definitive hematopoiesis, expressing Runx1 in the yolk sac blood islands at 7.5 dpc, establishing that intra-embryonic definitive HSC can originate from extra-embryonic primitive precursors in the yolk sac of the developing embryo.

As definitive HSC in the AGM mature, they migrate and enter the blood circulation [Christensen et al. 2004; Delassus and Cumano 1996] to colonize the liver [Morrison et al. 1995a]. In the fetal liver environment, such cells gain properties changing their identity from being nonproliferating, nondifferentiating and nontransplantable, to cells that can rapidly proliferate and self-renew to expand their

numbers to meet the growing requirements of the hematopoietic system [Ikuta and Weissman 1992; Lansdorp et al. 1993; Rebel et al. 1996b]. The capacity of the fetal liver HSC for multipotent blood cell production and long-term repopulation when transplanted into lethally irradiated hosts is extensive and unprecedented throughout ontogeny [Jordan et al. 1995; Rebel et al. 1996a; Rebel et al. 1996b]. With emergence of fetal liver hematopoiesis, the necessity of yolk sac erythropoiesis (12–14 dpc) decreases and leads to disappearance of yolk sac hematopoietic precursors. However, as yolk sac and fetal liver HSC display similar globin switching, these events are unlikely to be the result of alternating cell populations, but rather represent the outcome of developmental switches of primitive to definitive hematopoiesis and thereby changes in transcription. By the end of pregnancy, hematopoiesis in the liver transfers through the migration of HSC to the bone-marrow [Christensen et al. 2004; Delassus and Cumano 1996; Potocnik et al. 2000], which remains the main hematopoietic organ throughout life [Morrison et al. 1995b]. During these developmental processes, mesenchymal progenitor cells (with osteogenic, adipogenic and chondrogenic potential) in parallel home to and develop niches supporting self-renewal in the primary hematopoietic organs (fetal liver, bone-marrow and spleen) as these tissues develop in the fetus [Christensen et al. 2004; Mendes et al. 2005; Palis et al. 2001; Potocnik et al. 2000]. Whether the colonization of niches supporting hematopoiesis during midgestation is a multiwave process or the result of a constant flow of rare HSC in the fetal blood is currently unclear [Christensen et al. 2004; Delassus and Cumano 1996; Potocnik et al. 2000]. It appears likely that low numbers of HSC are constantly circulating both before and after their expansion and maturation in the fetal liver, until a suitable environment for hematopoiesis has developed in the bone-marrow.

The distinct developmental fates of extra- and intra-embryonic progenitors in extra- and intra-embryonic niches [Matsuoka et al. 2001; Orkin and Zon 2002; Walker et al. 2001; Yoder et al. 1997] thus require intrinsic developmental switches that alter lineage priming, gene expression, function, cell cycling and the phenotype of HSC. As transplantation of primitive extra-embryonic progenitors to intra-embryonic sites directs these cells to adopt a definitive fate, it appears as appropriate instruction is also contingent on environmental cues [Turpen et al. 1997].

3.2 Fetal to Adult Switch

Both embryonic and adult hematopoiesis is hierarchical, with differentiation occurring through distinct and sequential progenitor subsets [Kondo et al. 2003]. This suggests that the molecular mechanisms underlying cell fate decisions are conserved from embryo to adult. Despite of this similarity, the properties of fetal liver HSC that migrate to the bone-marrow by the end of gestation are in many aspects different from when they have adopted adult properties and fates in the bone-marrow [Jordan et al. 1995; Rebel et al. 1996a; Rebel et al. 1996b]. This fetal to adult developmental switch occurs in the bone-marrow during the first weeks after birth in

a precise manner and involves coordinated alterations in the abilities to self-renew, proliferate, differentiate as well as in regulation of gene expression, suggesting cell intrinsic regulation rather than random environmental changes [Bowie et al. 2007b; Kikuchi and Kondo 2006].

Expansion of HSC numbers in the fetal liver occurs with cell cycle kinetics that are significantly different from those of adult HSC in the steady state bone-marrow [Nygren et al. 2006]. Following lodging to the bone-marrow environment, there is a need for reduction of the extended proliferative activity and hence switching of the regulatory circuitry into a more quiescent state [Bowie et al. 2006]. Such transformation seems to occur during neonatal week three to four after birth and completely alters the cycling behavior from a fetal high proliferative into an adult quiescent state that is maintained throughout adulthood [Bowie et al. 2006]. Fetal liver HSC have a dominating lymphoid potential [Morrison et al. 1996], differ in the factor dependence for their differentiation compared to adult HSC [Kikuchi and Kondo 2006] and support differentiation to B- and T-cell subtypes that are distinct in function and phenotype and normally not present in adults [Hayakawa and Hardy 2000; Ikuta et al. 1990]. These differences in lineage priming are changed upon adoption of adult HSC properties during the first week after birth. Whether the effects of these switches are reversible remains to be determined, but as HSC of fetal type undergo similar changes with analogous kinetics following transplantation into an adult environment they must involve intrinsic and, at least during steady state conditions, irreversible changes of the fetal HSC [Bowie et al. 2007b]. This change could involve components of c-kit signaling [Bowie et al. 2007b], in accordance with the role of the c-kit receptor in control of self-renewal in HSC [Bowie et al. 2007a]. Shorter cell cycle passage time of fetal HSC might be due to intrinsic proliferative control that favors symmetric self-renewing cell divisions. Similar intrinsic changes occurs in otherwise quiescent adult HSC that are exposed to an environment that demands a high degree of self-renewing cell divisions, for instance following serial transplantation [Allsopp et al. 2001]. That self-renewing cell divisions dominates fetal HSC cell divisions is not only reflected by an increased proliferative activity in vivo compared to adult bone-marrow HSC but also an enhanced capacity to long term repopulate the blood system of lethally irradiated recipient mice [Rebel et al. 1996a]. This can not be correlated with differences in the ability of transplanted cells to migrate to and engraft in the host bone-marrow, as fetal HSC transplanted together with a population of competitive adult counterparts maintain this property. Cells from both origins engraft similarly well in the adult recipients but the fetal donor cells out competes adult cells over time. Although fetal HSC transferred to an adult environment undergo developmental switching into an adult state, they maintain an enhanced capacity to proliferate with extended and preserved self-renewing abilities compared to adult cells having developed normally [Bowie et al. 2007b; Bowie et al. 2006; Rebel et al. 1996a]. As transition into an adult stage seems to occur with precise timing, it appears as cell autonomous molecular switches tightly regulate lineage priming during fetal and adult hematopoiesis, but depend on proper development with sequential changes of fate that cannot be sidestepped.

3.3 The Adult to Old Switch

Upon the fetal to adult developmental switch, HSC persists throughout adulthood in a relatively quiescent state compared to their progeny [Nygren et al. 2006], with more or less maintained properties and regulatory control. However, randomly occurring events impose direct wear and tear on HSC during their life span and as such changes can accumulate over time due to the self-renewal capability of HSC, it appears reasonable that they can result in phenotypic and functional changes and even a reduced capacity to maintain cellular homeostasis and survival. However, gene expression patterns of aged HSC have revealed that, compared to post-mitotic cells in other tissues, stress induced damage does not seem to play a major role on HSC aging in steady-state [Rossi et al. 2005]. This might be a result of a unique ability of HSC to maintain a quiescent state relative to their down stream progeny throughout ontogeny [Nygren et al. 2006] which would allow HSC to escape from much of the negative stress associated with life long and continuous proliferation. Furthermore, not all observed changes of HSC during aging appears to be attributed to macromolecular damage, suggesting contributions of cell autonomous changes through internal molecular switches [Rossi et al. 2007b]. In line with this, loss of immune function of aged HSC is likely due to cell autonomous changes that results in altered gene expression favoring myeloid specific genes, resulting in lineage skewing towards a myeloid fate [Rossi et al. 2005]. Thus, reduced competence of the adaptive immune system appears to be a result of an increased myeloid progenitor cell capacity, at the expense of lymphoid developmental potential. Phenotypical and functional evaluations have shown that the numbers of HSC in the aged bone-marrow are significantly increased [Rossi et al. 2005; Sudo et al. 2000]. Transplantation of HSC from young and old donors into young recipients showed that the elevated numbers of HSC in old mice were due to cell autonomous changes in the HSC leading to a higher incidence of self-renewing cell divisions with HSC aging.

Taken together, HSC that until recently were assumed to be exempt from aging show progressive alterations in many aspects upon reaching advanced age. Such changes however does not seem to occur in an as controlled and defined way as for the developmental switches that occur during early development. Although the cellular changes act in a cell autonomous manner, the actual switching of a cell from young to adult might indeed be triggered by environmental cues. Such factors would be linked to aging of the cellular environment of HSC, thereby altering the regulatory circuitry that controls on demand production of HSC progeny and in maintenance of their own quiescence. In support of this, aged HSC have been suggested to be affected by changes of the interaction with the supporting HSC niches in the bone-marrow, with a reduced ability for adhesion to stromal cells, impaired homing to the bone-marrow following transplantation and increased responsiveness to mobilizing factors [Liang et al. 2005; Xing et al. 2006]. Thus, intrinsic and environmental changes that occur within the pool of HSC collectively sets the prerequisite for cellular deterioration. With this interpretation, the developmental history of each individual cell establishes how and when the aging process will begin.

Such processes seem to be irreversible and unaffected by environmental factors once initiated, as the transfer of aged HSC to a young environment does not change their adult identity [Rossi et al. 2005; Sudo et al. 2000].

4 Aging of Hematopoietic Stem Cells

Aging are generally though of as the sum of the deteriorative effects on a cellular identity or function that accumulates over time and eventually reaches a state that leads to tissue failure. However, for the hematopoietic system it is doubtful whether the consequences of aging are attributed to changes of the actual HSC due to accumulation of wear and tear or environmental signals. Instead, many changes on function and identity of HSC might in fact constitute normal developmental steps that occur through controlled and evolutionary conserved switches. Such adaptation to changes in environment and requirement imposed in the system, begin in the developing embryo and continue throughout ontogeny.

4.1 Aging as the Result of Environment

As described earlier in this chapter, HSC are exposed to dramatically different environments during their maturation. Commitment towards hematopoiesis occur outside the embryo in the yolk sac blood islands, transit through the AGM region and fetal liver for developmental switching and expansion, and by the end of fetal development find their home in the bone-marrow where they, except from rare and transient migration into the blood system for relocation, stay throughout adult life. During this process, wear and tear of the most primitive HSC do occur but the extent of this and the importance it plays for their function remains largely unknown. Throughout adulthood, it has been proposed that the main site that supports maintenance of HSC is the endosteal bone-marrow niche, providing a sanctuary with limited damage to proteins, membranes and DNA imposed by the environment. The cell layers that comprise the endosteal zone are highly hypoxic, suggesting that it provides an environment with low pressure of cellular damage derived from reactive oxygen species [Parmar et al. 2007]. Furthermore, hypoxic conditions might be important for optimal stimulation of self-renewal and quiescence and thereby play an important role in avoiding accumulation of cellular damage by limiting proliferation [Tothova et al. 2007]. Hypoxic conditions could be entertained by a low flow of extra-cellular fluids in the HSC niche, thereby also limiting exposure to toxins, metabolic by products and toxic compounds from immune responses as seen elsewhere in the organism.

As HSC by definition persist for the lifetime of an organism, developmental marks should be accumulating and with time reach levels influencing cellular function thereby imposing changes on HSC stem cell identity. Recent studies have however

underscored that environmental influences plays a minor role in the specification of these cells as transferring of aged cells to an young environment does not alter their identity or function [Rossi et al. 2005]. This argues for that environmental cues can not alter cell intrinsic changes of HSC once established or modify the genetic control that regulate the kinetics of this process [Phillips et al. 1992]. However, environmental factors such as toxins, inflammatory cytokines and DNA interfering compounds might well act over long time to impose some of the cellular changes accompanying age. Many of these are closely linked to metabolic and proliferative activities, emphasizing the importance of avoiding such influence on HSC by maintaining quiescence at all times, including during stress responses and extensive self-renewal [Nygren et al. 2006]. As all other cells, the bone-marrow stromal cells that constitute the HSC microenvironment age and undergo changes that might affect its HSC supporting capacity [Liang 2005 #646]. An increasing number of senescent stromal cells might be one important factor that through deteriorated HSC supportive function fails to prevent or possibly by themselves drive HSC aging.

4.2 Avoiding Aging by Quiescence

Downstream progenitor cell populations that have committed to individual hematopoietic lineages consist of a limited number of clones with an extensive capacity to produce mature effector cells of each lineage. The high degree of multiplying and differentiating cell divisions that take place within these populations impose a high degree of divisional stress on each cell. However, as progenitor cells lack extensive self-renewing properties, any damage on cells or their genetic material can only spread within the progeny of that clone and will disappear as it reaches its proliferative limit [Hayflick 1965; Lemischka et al. 1986]. In contrast, HSC, which through their asymmetric cell divisions can generate all different progenitor cell subsets, must find means to avoid such accumulation of damage as they will be carried on and amplified at progenitor cell stages for the rest of the life of that HSC clone. It therefore appears likely that HSC have adapted a regulatory circuitry that limits proliferation at the HSC stage.

In vivo labeling with the thymidine analogue BrdU into the DNA of proliferating cells have established that although all HSC constantly divide and participate in hematopoiesis, they are generally quiescent, with a population turnover that is distinguishable from that of the downstream progenitor cells even during stress or extensive expansion [Bradford et al. 1997; Cheshier et al. 1999; Nygren et al. 2006]. To expand the numbers of progeny generated from each clone, a finite but extensive proliferative capacity instead occurs at downstream progenitor cell levels. Thus, clonal stability of rare HSC seems to be the general incidence, whereas clonal succession occurs within all hematopoietic progenitor cell populations as each progenitor cell has a limited life span and is eventually succeeded by a new progenitor cell.

When studying hematopoiesis during aging, any cell type will however eventually reach its end point and succumb or perhaps enter senescence. As new HSC

clones are not formed after midgestation embryogenesis [Gothert et al. 2005] loss of HSC clones can not be replenished but only substituted by expansion of similarly old clones that will eventually reach senescence or die. Senescent cells are metabolically active but have a changed pattern of gene expression and an irreversible loss of proliferative capacity. HSC senescence should normally represent a minor problem, if occurring at all, as HSC can be propagated for as long as four times the normal life span of a mouse through serial transplantation into sequential recipients [Harrison 1979]. The elements that are involved in avoiding senescence of HSC are not known but through studies of different mice strains, several genetic elements has been identified that are indispensable for controlling proliferation and thereby preservation of the HSC pool [Chen et al. 2000]. Although HSC appears to have found means to escape from proliferative stress such as through lodging to a hypoxic environment with low degree of oxidative stress [Parmar et al. 2007; Tothova et al. 2007], efficient exclusion of toxic components taken up from the environment by multidrug resistance membrane transporters [Zhou et al. 2002] and limiting genetic deterioration from repeated explicative stress [Nijnik et al. 2007; Rossi et al. 2007a], complete protection from these effects is unlikely. An example of this is the shortening of the critical telomere elements at the ends of the chromosomes following extensive proliferation.

Telomeres are the regions of highly repetitive DNA at the end of the chromosomes and functions as a disposable buffer of genetic material. Every time chromosomes are replicated, DNA polymerase complexes are incapable of continuing replication all the way to the end of the DNA strand of each chromosome, thereby leading to a gradual loss of nucleotides with each mitosis. This ultimately results in a genome with an increased chromosomal instability and, as a cellular defense mechanism, an increased predisposition to enter senescence or cell death. In some cells, including somatic stem cells, telomerase extend telomeres by adding extra repetitive DNA elements constituting telomeric sequences. However although genetic instability and functional impairment of HSC coincides with telomere shortening following extensive proliferative stress and following genetic alteration of telomerase function [Allsopp et al. 2001; Allsopp et al. 2003a; Samper et al. 2002], maintenance of telomeres is likely not sufficient by itself to avoid stem cell exhaustion [Allsopp et al. 2003b].

4.3 Avoiding Aging by Asymmetry

The quiescent stem cell specific asymmetric partitioning of cell fate during cell division of HSC into either preserved stem cell identity, or loss of such and commitment towards differentiation, limits the proliferative tension on the stem cell clone by allowing one of the daughter cells to maintain quiescence. Which elements that are unequally distributed to the daughter cells and how such partitioning takes place is not fully understood. However, displacement from a certain stem cell supporting environment within the niche might play a role [Kiger et al. 2001; Tulina and Matunis 2001; Wilson and Trumpp 2006] as well as regulated

or stochastic distribution of transcription factions, organelles, receptors and other cellular constituents and biomolecules into gradients that establish differences in cell fate [Arai et al. 2004; Rusan and Peifer 2007; Yamashita et al. 2007; Zhang et al. 2003]. It would therefore seem reasonable that partitioning of cell fates by asymmetric cell divisions might play a role for avoiding aging of the HSC clone that strive for persistence throughout life. During stem cell divisions, centrosomes can be asymmetrically inherited with mother centrosomes that always ends in the daughter cell with remained stem cell identity, arguing that also genetic material could be passed on from stem cell to stem cell in a similar fashion. The concept of such partitioning of the DNA have been hypothesized and could explain how long lived cells that proliferate extensively avoid accumulation of genetic defects acquired during replication [Nijnik et al. 2007] by using the same DNA strand as template for DNA polymerase replication and passing it along into the daughter cell that inherit stemness, whereas the newly replicated strand ends up in the daughter cell with lost stem cell capacity [Cairns 1975]. Retaining the same set of template DNA strands throughout development could help preventing adult stem cells from accumulating mutations arising from errors in DNA replication. Instead, randomly occurring mutations during replication within a HSC clone are passed on to nonstem cell daughters that soon terminally differentiate. Such mechanism would reduce the rate of accumulation of mutations in HSC that would otherwise eventually lead to serious genetic disorders or aging [Rossi et al. 2007a]. The idea of an immortal strand in HSC has however to be strengthened by experimental data, and if present, it has to be established how such stem cell specific partitioning of immortal template and mortal copy strands take place. All definitive HSC clones are established during midgestation embryogenesis and thereafter no new clones are born. Thus expansion of the HSC pool thereafter can only occur through multiplication of such HSC clones. Depending on when immortal templates would be established, not all HSC necessarily need to have the capacity to partition DNA into mother and daughter cells following replication. Similarly, asymmetric distribution of proteins and cellular constituents, an event that plays important roles in specifying asymmetry of the daughter cells from asymmetric cell divisions [Enver et al. 1998; Takano et al. 2004], could be speculated to also be operational to specify fates at a HSC level, although to date representing an under explored mechanisms to these processes.

Chromatin remodeling is critical for regulating transcription, replication, recombination and segregation of the chromosomes. Histone complexes organize chromatin and play a major role in epigenetic imprinting of the DNA, and a critical role in HSC biology for the polycomb protein Bmi-1, a transcriptional repressor that maintain repression of genes after a cell division and thereby maintain epigenetic memory, was suggested [Lessard and Sauvageau 2003; Park et al. 2003]. It appears likely that such processes play key roles in determination of stemness of daughter cells from self-renewing HSC cell divisions, although the order of such events remain currently unknown. However, as epigenetic imprinting of DNA has to be replicated onto the daughter chromatin following each cell division, it appears reasonable that accumulated numbers of cell divisions might lead to functional changes, including reductions in self-renewing ability.

4.4 Genetic Control of Aging

Aging is a multi parameter sequence of events controlled by both environmental factors and the genetic composition of the individual. As outlined above, environmental factors can influence on normal wear and tear of HSC and their development, but seem to have a subordinate role for establishing the transcriptional networks that specifies developmental switches and aging. Although the molecular mechanisms have not been elucidated in detail, it is clear that various genetic traits are involved in the modulation of life span [de Haan et al. 2002; Geiger et al. 2005; Rossi et al. 2007a]. Such modulation in HSC involves reduction of damage to the cell and its genetic components over time, and is crucial to avoid premature ageing and senescence. Damage control is not mainly supplied by repair systems that maintain the integrity of proteins, DNA and cellular components, but also accomplished through preventing metabolic stress and genetic instabilities acquired from extended divisional activity. This provides a strategy to limit the number of deleterious actions occurring within the limited and irreplaceable HSC pool, and is mainly accomplished by an in nature unique ability to constantly participate in the process of hematopoiesis, while still maintaining proliferative quiescence over time [Nygren et al. 2006].

Several genes involved in maintaining HSC quiescence and the action of their protein products have been characterized. These components form a network that signals to the stem cell to maintain stem cell properties through self-renewing cell divisions [Reya et al. 2003; Willert et al. 2003] and is believed to transfer epigenetic memory that identifies stemness to at least one of the daughter cells [Lessard and Sauvageau 2003; Park et al. 2003]. The process of self-renewal however needs to be restricted and factors are needed that interfere with cell cycle control to restrict proliferation [Hock et al. 2004] and maintain and strengthen adhesive interaction of the HSC with the niche supporting quiescence [Wilson et al. 2004; Yang et al. 2007]. However, although many components of the circuitry that signals self-renewal and quiescence in the HSC and their niche have been postulated, little direct mechanistic insights have so far been provided.

Genes and genetic elements that have a general effect on the life span of an organism suggests that molecular machineries can be activated to ensure that homeostasis is maintained at various situation of stress, thereby putting some sort of break to the aging process. Genetic information located in the mitochondria has been identified as one major heritable component involved in aging. Instabilities of the mitochondrial DNA due to damages or replication can lead to mitochondrial dysfunction, increased oxidative stress and cellular senescence emphasizing the importance of long lived cells to limit metabolic activity and proliferation [Cadenas and Davies 2000]. Studies of cellular processes in mice have been standardized to involve only a limited number of inbred mouse stains. In these a varying degree of effects on HSC function have been observed during aging, however generally HSC does not seem to loose regenerative capacity as they age [Rossi et al. 2005]. This is further supported by studies demonstrating maintenance of hematopoiesis during conditions and time spans that by far extends the normal life expectancy of the organism [Ross et al. 1982].

5 Conclusion

The constant ongoing process of cell replacement in the hematopoietic system imposes a strong degenerative stress on the cellular elements that participate in these processes. Luckily, many of these adverse effects from prolonged regeneration disappear upon cellular differentiation and are therefore present only transiently. However, as stem cells are maintained throughout life, accumulation of any damage or other heritable cellular traits that occurs along these processes and its regulatory networks will have dramatic effects due to the hierarchical structure of hematopoietic development. Limiting metabolic and divisional stress in hematopoietic stem cells is therefore key for maintaining their appropriate lifelong function and to avoid proliferative exhaustion or leukemic transformation with age.

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Genetics

Associations of Cytokine Polymorphisms with Immunosenescence

Elissaveta Naumova and Milena Ivanova

Contents

Abstract: Deterioration of the immune system with aging is associated with an increased susceptibility to infectious diseases, cancer and autoimmune disorders. It has been demonstrated that immunosenescence is associated with chronic, low-grade inflammatory activity. The aging process is very complex and longevity is a multifactorial trait, which is determined by genetic and environmental factors, and the interaction of "disease" processes with "intrinsic" ageing processes. It is hypothesized that the level of immune response as well as possibly longevity could be associated with genes regulating immune functions. It is further hypothesized that the diversity of these genes might influence successful aging and longevity by modulating an individual´s response to life-threatening disorders. Several studies have focused on the role of genes encoding molecules with immune functions. In this chapter we will review the data on the role of cytokine gene polymorphisms in human longevity.

Keywords: Cytokines • Cytokine gene polymprphisms • Immunosenescence Longevity

E. Naumova (✉) · M. Ivanova
Central Laboratory of Clinical Immunology
University Hospital "Alexandrovska"
1.G. Sofiisky str., 1431 Sofia, Bulgaria
Tel: +3952 9230 690
Fax: +3592 9230 496
E-mail: immun@alexandrovska-hospital.bg

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1 Introduction

Aging is a universal phenomenon affecting all animal species. This physiological process could be characterized as: inevitable consequence of being a multicellular organism; associated with a random passive decline in function; leading to a global loss of homeostasis over time and mortality increasing with aging (Helfand and Rogina, 2003). Aging is determined by a complex interaction of genetic, epigenetic and environmental factors, but a strong genetic component appears to have an impact on survival to advanced age. Among the several theories of aging proposed, the genetic theory suggests that several genes are involved in longevity (Kirkwood, 2002; Browner et al. 2004). Additionally, studies of Mitchell et al. indicated that up to 25% of the variation in human lifespan is heritable (Mitchell et al. 2001). The genetics of human longevity is quite peculiar in a context where antagonistic pleiotropy can play a major role and genes can have different biological role at different ages. Data of several studies imply that aging process may be associated with alterations in the immune system, suggesting that genetic determinants of senescence also resides in those polymorphisms for the immune system genes that regulate immune responses. Genes, encoding molecules involved in the development of protective immunity are highly polymorphic, present significant variation possibly resulting from an evolutionary adaptation of the organism facing an ever evolving environment. These genes include: HLA genes; genes encoding "unusual" HLA-like molecules (CD1); killer cell immunoglobulin-like receptor genes (KIR); leukocyte Fcγ receptor genes; cytokine and cytokine receptor genes; Toll-like receptor gene family; TNF- receptor associated factors. Several studies have focused on the role of cytokine gene polymorphisms for human longevity. The diversity of these genes might influence successful aging and longevity by modulating an individual´s response to life-threatening disorders.

In this chapter we summarize present knowledge on the role of cytokines in human longevity. Cytokines are an internal part of the immune response stimulated by antigen presentation in the context of HLA. Many studies have shown that the pathology of some infectious, autoimmune and malignant diseases is influenced by the profiles of cytokine production in pro-inflammatory (Th1) and anti-inflammatory (Th2) T cells. Additionally some authors have shown differences in cytokine levels in elderly and possible association with age-related diseases. Pro-inflammatory cytokines play a role in chronic inflammation, a phenomenon proposed to call "inflammaging" (Salvioli et al. 2006). People genetically predisposed to develop weak inflammatory activity seems to have fewer chances of developing cardiovascular diseases and subsequently live longer if they do not become affected by serious infectious diseases. Ferrucci et al. 1999, Harris et al. 1999 demonstrated that increased IL-6 serum levels could be a marker for functional disability and predictor of mortality in elderly. Increased expression levels of IL-6 were observed also in stress conditions, one of the characteristics of ageing (Heinrich et al. 2003). Elevated levels of this cytokine associated with development of frailty and susceptibility to diseases in elderly were also observed (Forsey et al. 2003). This inflammatory

marker could be involved in low-grade inflammatory that develops with age (Cohen et al. 1997; Bruunsgaard et al. 1999; Ershler and Keller 2000). Dysredulation of IL-6 has been thought to be involved in the pathogenesis of a variety of age-related diseases, such as diabetes and atherosclerosis, which have a substantial inflammatory pathogenesis (Chamorro 2004; Dandona et al. 2004). IL-8 is also considered to be a potent inflammatory agent. However, IL-8 has also been reported to serve as an organ protective factor. It seems possible that an association of an increased serum level of IL-8 and a low level of IL-6 is related to longevity (Wieczorowska-Tobis et al. 2006). Additionally in elderly was observed a decreased capacity to produce IFN-γ, IL-2, IL-4 upon stimulation (Franceschi et al. 2000). The higher levels of TNF-α also correlate with functional status and decreased chance of long-life survival in elderly (Ferrucci 1999; Harris et al. 1999; Ershler et al. 2000; Forsey et al. 2003). Moreover, dysregulation and, in particular, overproduction of TNF has been implicated in a variety of human diseases including sepsis, cerebral malaria, and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis (RA), systemic lupus erythematosus, and Crohn disease, as well as cancer. Interestingly, in a very large

Table 1 Gene polymorphisms of pro-inflammatory cytokines associated with aging

Cytokine gene polymorphism	Effect	Population	References
IL-2 (-330 T/C)	No association in elderly	Irish	Ross et al. 2003
	Increased (T-low) marginally in centenarians	Italian	Scola et al. 2005
IL-6 (-174 C/G)	Increased (C-low) in male centenarians	Italian	Bonafe et al. 2001
	No association	Sardinia, Southern Italy	Capurso, 2004; Pes et al. 2004
	Decreased (G/G-high) in octogenarians and nonagenerians	Irish	Rea et al. 2003; Ross O et al. 2003
	No association in nonagenarians	Finish	Wang X et al. 2001
	Increased G allele in elderly survivors	Finish	Harume et al. 2005
	Increased (GG) in elderly	Danish	Christiansen L et al. 2004
	No association	Bulgarian	Naumova et al. 2004
IFN-G (+874 T/A)	Increased (T/T-high) in female centenarians	Italian	Lio D et al. 2002
	No associations	Bulgarian	Naumova et al. 2004
TNF-A (-308 G/A)	Decreased (A-high) in centenarians	Danish	Buunsgaard H et al. 2004
	No association in nonagenarians	Finish	Wang X et al. 2001
	No association in centenarians	Italian	Lio D et al. 2003
	No association in elderly	Bulgarians	Naumova et al. 2004

study in Italian population IL-1Ra plasma levels were increased with age in both male and female subjects (Cavallone et al. 2003). Because of the pivotal role of anti-inflammatory cytokines TGF-β1 and IL-10 in regulation of immune responses, the variability of their levels may affect low grade inflammation that develops with age. It has been shown that the elevated level of anti-inflammatory cytokines IL-10 and TGF-β in serum of elderly is associated with increased resistance against septic shock (Forsey et al. 2003). Increased ex vivo capacity of macrophages from elderly to produce anti-inflammatory IL-10 was also found. Intriguingly, the existence of "risk immunological phenotype," probably associated with lack of tight control in systemic inflammation was also discussed.

Similarly to other genes, coding molecules with immune functions, cytokine genes are highly polymorphic. Most of the polymorphic sites identified so far are located in the noncoding regions, containing regulatory sequences, while exon sequences are highly conserved. Three main forms of polymorphisms were identified in cytokine genes: single nucleotide polymorphisms (SNPs) (Kruglyak et al. 1999), variable number of tandem repeats and micro-satellites (Weber and May 1989; Bidwell et al. 2001). Although still controversial, polymorphic variants observed for some cytokine genes have been correlated to the level of gene expression. Thus the cytokine gene polymorphism may be responsible for observed inter-individual differences in cytokine production and may be one possible mechanism for perturbation of the Th1/Th2 balance. Some polymorphisms may have a functional significance by altering directly or indirectly the level of genes expression and/or its function, others may only be useful for the determination of genetic linkage to a particular haplotype associated in turn with a given clinical condition (Bidwell et al. 1999, 2001).

Although the data are limited and controversial (Caruso et al. 2000) and many discrepancies are reported likely due to population-specific interactions between gene pool and environment, interleukins could be considered as putative "longevity genes. " It has been hypothesized that longevity could be associated with cytokine gene polymorphism correlated with different level of cytokine expression and modulating immune response to several diseases (Ershler et al. 2000; Bruunsgaard et al. 2001; Volpato et al. 2001). Taking into account the internal part of cytokine genes in immune response, the regulation of cytokine expression level and their polymorphic nature, investigation the genetic variations of these loci with functional significance could be appropriate immunogenetic candidate markers implicated in the mechanism of successful aging and longevity.

Genetic variations correlating with elevated levels of pro-inflammatory cytokines have been negatively associated with ageing (Bhojak et al. 2000). Several studies showed that cytokine polymorphisms related to different level of secretion were associated with longevity. Genetic polymorphisms, associated with high level of IL-10 expression were increased (Lio et al. 2003), while polymorphisms possibly related to increased expression of proinflammatory cytokines - IFN-γ, TNF-α and IL-6 were decreased in elderly individuals (Lio et al. 2002; Bruunsgaard et al. 2004). These data confirmed the hypothesis that longevity is related to antiinflammatory genotype profile. Additionally, the pro-inflammatory cytokine profile was

correlated with decreased life span in elderly. However, in elderly with different ethnical background, investigations reported contradicting results on associations with cytokine gene polymorphism (Bonafe et al. 2001; Lio et al. 2002). Additionally, the majority of data were associated with investigation of single polymorphisms in single cytokine genes. The analysis of extended haplotypes which include several polymorphisms in the cytokine gene, as well as haplotypes which consist of SNPs in different cytokine genes will help to determine the precise immunogenetic basis of longevity.

2 Gene Polymorphism of Proinflammatory Cytokines and Aging

2.1 IL-2

IL-2 is a proinflammatory cytokine, which plays a central role in activation of T-cell mediated immune response and defects in IL-2 mediated activation induce severe immune deficiency (Demoulin and Renauld, 1998). Several polymorphisms in the promoter (position -330) and coding (position +166 and exon 1) regions were described in IL-2 gene. A promoter polymorphism -330 T/C was shown to influence IL-2 production in anti-D3/CD28-stimulated peripheral blood lymphocytes. T-lymphocytes from -330 CC homozygous subjects are able to produce higher amount of IL-2 than heterozygous or -330 TT homozygous individuals (Hoffmat et al. 2001).

Age-related decline in IL-2 production has been recognized since the early work of Gillis et al. Subsequent studies showed that IL-2 is reduced in aged subjects with associated effects on intracellular activation on nuclear transcription pathways (Rea et al. 1996; Pawelec et al. 2002). In humans, high IL-2 serum levels characterize subjects affected by Alzheimer's disease. Resent study in Italian (Scola et al. 2005) and Irish (Ross et al. 2003) elderly subjects did not showed a statistically significant effect of IL-2 -330 polymorphism in aging. However, a T allele associated with IL-2 low producer genotype was discussed to be marginally associated with aging (Scola et al. 2005). Data suggested that the genetic background favoring an increased IL-2 production might be detrimental for longevity.

2.2 IL-6

IL-6 is a pleotropic growth factor involved in different physiological and pathological processes. IL-6 is considered to be a potent inflammatory agent. It was found also to inhibit neutrophil apoptosis, suggesting that there is an autocrine or paracrine antiapoptotic role for IL-6 (Lindermans et al. 2006).

The human IL-6 gene is mapped to chromosome 7p21–24. Different studies identified three SNPs (−597G/A, −572G/C и −174G/C) and one AT polymorphism (−373(A)n(T)m) in 5′ regulatory region in the gene (Fishman et al. 1998; Terry et al. 2000; Georges et al. 2001). It was demonstrated that IL-6–174C allele was significantly associated with lower plasma concentrations of this cytokine (Terry et al. 2000).

Despite of the significant number of studies on possible role of IL-6 gene polymorphisms in different diseases, the associations still remain to be clarified (Rauramaa et al. 2000; Terry et al. 2000; Humphries et al. 2001; Nauck et al. 2002). Great amount of papers reported a positive association between some polymorphic markers of IL-6 gene and human longevity, and capacity of producing low levels of IL-6 thought life-span appears to be beneficial for longevity (Wright et al. 2003; Christiansen, 2004; Franceschi et al. 2005; Hurme et al. 2005) Most studies focused on IL-6 -174 C/G polymorphism and susceptibility to common causes of morbidity and mortality among elderly, such as type 2 diabetes, cardiovascular diseases, and dementia. IL-6-174 C/G polymorphism is predictive for longevity (Salvioli et al. 2006) Data on centenarians and elderly individuals from Italy showed increased frequency of C alleles in male centenarians and it seemed to be a gender specific effect on longevity (Bonafe et al. 2001). Correlations with the serum levels showed that men carrying the GG genotype had higher IL-6 serum levels in respect to subjects with CC or CG genotypes. The authors hypothesized that individuals predisposed to produce high level of IL-6 (men carrying GG) have a reduced capacity to reach the extreme limits of human life-span. Additionally, authors demonstrated that age-related increase of IL-6 serum levels in women is quite independent from -174 C/G genotype. It has also been shown that the proportion of IL-6 high producers (GG genotypes) was increased by individuals affected by age-related diseases with inflammatory pathogenesis—diabetes, atherosclerosis, osteoporosis, and neurodegenerative diseases. Similarly, Rea et al. (2003), Ross et al. (2003) reported decreased frequency of IL-6 -174 GG carriers in Irish octogenarian and nonagenarian subjects from the BELFAST elderly longitudinal ageing study. However, in Finish nonagenerians analysis on IL6-174, IL1a-889, IL1b-511, IL1Ra VNTR, IL10-1082, and TNFa-308 did not show any associations, alone or in combinations (Wang et al. 2001). It appears that IL-6 polymorphism does not affect life expectancy neither in the Sardinian population, nor in people from southern Italy, suggesting that the effect of IL-6 polymorphism on longevity might be population specific and dependent on gene – environment interactions (Capurso, 2004; Pes et al. 2004). Similar lack of association of IL-6 gene polymorphism with longevity was observed in the Bulgarian population (Naumova et al. 2004). These controversial data could be partly explained by population specific factors including genetic background, environmental factors and life stile. Most of studies analyzed the effect of isolated polymorphisms and this could partly contribute to the contradicting results. Recently, Terry et al. (Rauramaa et al. 2000) demonstrated that haplotype combination of promoter polymorphisms in IL-6 gene is more informative marker associated with the level of gene expression, compared with the influence of −174 G/C in isolation. Additionally, studies have demonstrated that CC or CG carriers have an increased risk of Alzheimer disease (AD) and cardiovascular diseases (Zhang et al. 2004).

2.3 IFN-γ

IFN-γ is one of the most representative type 1 cytokines, which plays a pivotal role in defense against viruses and intracellular pathogens and the induction of immune-mediated inflammatory responses. Taking into account the key role of this cytokine, its genetically controlled production is focused on investigation in several studies associated with longevity. Among numerous intronic polymorphisms in the IFN-γ gene, a variable length CA repeat sequence and polymorphism in the intron 1 at position +874 relative to the transcriptional start site has been implicated to influence the level of gene expression in vitro (Pravica et al. 1999). The single nucleotide polymorphism +874 T/A is one well-known single-nucleotide polymorphism at the 5' end of the CA repeat region in the first intron of the IFN-γ gene. Specific binding of the nuclear transcription factor-κB to the DNA sequence containing the +874 T allele has been reported and it could have functional consequences for the transcription of the IFN-γ gene and could then influence the rate of expression. Studies in Italian centenarians showed increased frequency of +874 T/T in female centenarians. On the other hand, investigations in elderly individuals from the Bulgarian population did not show significant differences in IFN-γ (+874) allele distribution compared to young controls (Naumova et al. 2004).

2.4 TNF-α

TNF-α is a pro-inflammatory cytokine involved in the immune response. This pleiotropic cytokine plays a wide variety of functions in many cell types.

The gene for TNF-α is located within the class III region of the major histocompatibility complex, which is a highly polymorphic region and its expression is tightly controlled at the transcriptional and posttranscriptional level. Several biallelic polymorphisms have been described within the TNF-α gene, including six in the promoter region at positions -1031T>C, -863C>A, -857C>T, -376G>A, -308G>A and -238G>A. Moreover, a number of studies have shown that the TNF-α promoter polymorphisms have a significant effect on transcriptional activity. Susceptibility to many diseases is thought to have a genetic basis, and the TNF gene is considered a candidate-predisposing gene. However, unraveling the importance of genetic variation in the TNF locus to disease susceptibility or severity is complicated by its location within the MHC and the strong linkage disequilibrium with other genes. Several investigations reported associations of MHC haplotypes with different TNF-α phenotypes: DR3 and DR4 haplotypes were correlated with high level of TNF-α (Jacob et al. 1990; Abraham et al. 1993), while DR2 haplotypes were associated with low expression (Bendtzen et al. 1988; Jacob et al. 1990). These finding proposed the existence of functional polymorphism involved in the regulation of TNF-α production. SNPs at position -308 have been commonly studied with respect to their influence on TNF-α expression. Transfection studies

Table 2 Gene polymorphisms of anti-inflammatory cytokines associated with aging

Cytokine gene polymorphism	Effect	Population	References
TGF-B1 (915 C/G)	Decreased C allele and C/G genotype in centenarians	Italian	Carrieri G et al. 2004
TGF-B1 (cdns 10, 25)	No associations in elderly	Bulgarians	Naumova et al. 2004
IL-10 (-1082 A/G)	Increased (G/G-high) in male centenarians	Italians	Lio D et al. 2002
	No association with longevity	Finish	Wang X et al. 2001
	No association with longevity	Irish	Ross O et al. 2003
	No association with longevity	Sardinian	Pes G et al. 2004
IL-10 (-819 C/T)	No association with longevity	Italian	Lio D et al. 2002
IL-10 (-592 C/A)	No association with longevity	Italian	Lio D et al. 2002
IL-10 (-1082G,-819C,-592C)	Increased in elderly	Italian	Lio D et al. 2002
IL-10 (-1082G,-819C,-592C)	Increased in elderly	Bulgarians	Naumova et al. 2004
IL-10 (-1082A,-819T,-592A)	Decreased in elderly	Bulgarians	Naumova et al. 2004

in human B-cell lines showed that the presence of rare TNF2 allele (A at position -308) results in higher constitutive and inducible levels of TNF expression compared with a common TNF1 allele (G at position -308), confirming the importance of this site in the transcriptional regulation of the TNF gene (Wilson et al. 1997; Makhatadze et al. 1998; Lio et al. 2001; Hajeer, 2001). The functional relevance of this SNP has been confirmed by its involvement in determining susceptibility to immune-inflammatory diseases (Makhatadze, 1998; Lio et al. 2001; Hajeer and Hutchinson 2001; Dalziel et al. 2002; Heijmans et al. 2002; O'Keefe et al. 2002; Sakao et al. 2002; Witte et al. 2002). Although the polymorphism -308 G/A associated with different gene expression is one of the most widely investigated in different diseases, no correlation of this SNP and longevity was found in centenarians from the Finnish and the Italian population (Wang et al. 2001; Lio et al. 2003). Similar results were observed for elderly individuals from the Bulgarian population (Naumova et al. 2004). In the Danish population, however decreased frequency of A allele was observed among centenarians. Positive association with the process of successful ageing was observed also in men centenarians from Italian population when the two SNPs -308 G/A from TNFA gene and -1082 G/A SNP from IL10 gene were analyzed simultaneously (Wang et al. 2001). The group of Lio D et al. reported that an anti-inflammatory genotype TNFA GG (low)/ IL10 GG (high) has a protective role in longevity. TNF-A and IL-10 have complex and opposing roles, and an autoregulatory loop appears to exist (Candore et al. 2002).

3 Gene Polymorphism of Antiinflammatory Cytokines and Aging

3.1 TGF-B1

TGF-B1 is a multifunctional cytokine that regulates cell proliferation, differentiation, and migration, and it was considered as an aniinflammatory molecule (Wright et al. 2003). In the study of Carrieri et al, the plasma levels of biologically active TGF-B1 were significantly increased in the elderly group, independently from TGF-B1 genotypes (Carrieri et al. 2004). The TGF-β1 gene consists of 7 exons and 6 introns. Until now, eight polymorphisms in the promoter (-509 C→T, -800 G→A and -988 C→T), coding (+896 T→C, +915 G→C, +788 C→T, +652 C→T and +673 T→C) regions end one deletion (713 del C) in inton 4 of the TGF-β1 gene were discovered. Polymorphisms +896 (codon 10) and +915 (codon 25), associated with different level of expression are the most commonly studied. For polymorphism +915 G/C, the presence of C allele is generally associated with lower TGF- β synthesis in vitro and in vivo. Association between the presence of particular TGF-β1 allele and the level of the product indicates that the G-800 A and C-509 T polymorphisms may also be involved in the modulation of expression of the TGF- β1 gene. The -509 T allele - has been reported to be associated with marginally higher transcriptional activity of TGF- β compared to the -509C allele. TGF-β -800 G→A polymorphism is in a consensus CREB (cAMP response element-binding protein) shalf-site. The presence of A allele was suggested to have reduced affinity for the CREB family of transcription factors, resulting in a lower level of total TGF- β1 in the circulation. Analysis of these three SNPs +915 G→C, -509 C→T and -800 G→A by the group of Carrieri G et al. observed that only +915 C allele and GC genotype with significantly lower frequency, compared to controls. Additionally they found also decreased frequency of extended haplotype G -800/C -509/C 869/C 915 and elevated level of TGF-β1 in elderly, but correlation with investigated genotypes in TGF-B1 gene was not found (Carrieri et al. 2004). Similarly no associations of TGF-B1 codons 10 and 25 genotypes with longevity were observed in Bulgarians (Naumova et al. 2004). It has been hypothesized that genetic determined cytokine profiles of TGF-β1 could be involved in mechanism of successful ageing but more data are need to confirm this results.

3.2 IL-10

IL-10 is a powerful cytokine that inhibits lymphocyte repeication and secretion of inflammatory cytokines (My-Chan Dang et al. 2006). Since one of the main functions of IL-10 is to limit inflammatory responses (Moore et al. 2001), polymorphisms in the regulatory region of this gene could be possibly related to longevity. Stimulation of human blood samples with bacterial lipopolysaccharide showed

variation of IL-10 production, suggesting a genetic component of approximately 75% (Westendorp et al. 1997). Inter person differences in the regulation of IL-10 production may be critical with respect to the final outcome of an inflammatory response.

The IL-10 gene is located on chromosome 1 at q31–32. Several polymorphisms in the human IL-10 5' flanking region and two microsatelites associated with differential IL-10 production have been identified. The most extensively investigated SNPs are in the promoter region at position -1082, -819 and -592 (Turner et al. 1997, D'Alfonso et al. 2000; Kube et al. 2001) correlating with different transcriptional activity. The three dimorphisms appear in three potential haplotypes: GCC, ACC and ATA (G/A at position -1082, C/T at position -819 and C/A at -592 correspondingly) related to different level of gene expression. The ability of individuals to produce high levels of IL-10 is evidently controlled by a G at position -1082, as this variant is found in the highest producers (Turner et al. 1997; Eskdale et al. 1998; Crawley et al. 1999; D'Alfonso et al. 2000; Kube et al. 2001; Lio et al. 2002). Several studies reported the linkage between the sites -819 and -592. The A allele of the -592 SNP was found to be associated with lower stimulated IL-10 release. In the presence of allele -1082A, stimulation of lymphocytes with concanavalin A resulted in lower IL-10 production than in allele -1082A negative cells (Turner et al. 1997; Hutchinson et al. 1999). The functional relevance of this SNP has been shown by its involvement in determining susceptibility to immune-inflammatory diseases (Hajeer et al. 1998; Crawley et al. 1999; Tagore et al. 1999; Howell et al. 2001; Girndt et al. 2002; Shoskes et al. 2002; Wu et al. 2002) The two dimorphisms -819 and -592 exhibit strong linkage disequilibrium.

The IL-10 -1082 A/G polymorphism has been reported to be a male-specific marker for longevity (Lio et al. 2002), while no differenced were found regarding the -819 and -592 polymorphisms. The -1082GG genotype, associated with high IL-10 production, was argued to confer an anti-inflammatory status (Lio et al. 2002). Studies in Bulgarians demonstrated significant differences for two IL-10 haplotypes: one of them (-1,082A,-819T,-592A), possibly associated with the low level of gene expression was decreased in elderly, while the other (-1,082G,-819C,-592C) associated with high level of cytokine gene expression was significantly more frequent among healthy elderly compared to young controls. This effect was more pronounced in GCC homozygous individuals as indicated by the analysis of IL-10 genotypes. However, studies in two other populations— Irish nonagenarians (Ross et al. 2003) and Finish nonagenarians (Wang et al. 2001) did not show any association with longevity. A possible explanation for the negative results in the Irish and Finnish studies could be the younger age of the old subjects investigated in comparison with the Italian study. Interestingly to note that the IL-10 -1082 GG genotype is much less frequent in patients affected by Alzheimer's disease (Lio et al. 2003).

In summary, the capability to maintain a lower production of pro-inflammatory cytokines appears to be favorable for reaching the extreme limits of human life span in good health conditions and could be genetically controlled. Cytokine genes related to inflammation seems particularly relevant taking into account that the

innate immunity is more involved during inflammation, and a chronic inflammatory status appears to be the major component of the most common age-related diseases, including cardiovascular diseases and infections. The emerging data on the pivotal role of additional interactions in affecting expression of some relevant cytokines (for example zinc–gene interactions) and studies in populations with different environmental background will allow to clarify further the role of cytokines in aging.

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Cytokine Polymorphisms and Immunosenescence

Owen A. Ross, Kelly M. Hinkle and I. Maeve Rea

Contents

Abstract: The influence of genetics on immunosenescence is still to be resolved. Common genetic variants (polymorphism) that reside within the genes encoding cytokines are candidates to positively or negatively affect immunosenescence. Cytokines regulate the type and magnitude of the immune function. Polymorphism can influence the expression level of the cytokine protein which can subsequently cause an imbalance in the cytokine cascade.

Herein we examine the current literature with respect to cytokine polymorphisms in ageing and the age-related neurodegenerative disorder, Parkinson's disease.

O. A. Ross (✉) · K. M. Hinkle
Molecular Genetics Laboratory and Core
Mayo Clinic, Department of Neuroscience
4500 San Pablo Road, Jacksonville, FL 32224, USA
Tel: (904)-953-0963
Fax: (904)-953-7370
E-mail: ross.owen@mayo.edu

I. M. Rea
Department of Geriatric Medicine, School of Medicine
Dentistry and Biomedical Science, Queens University
Belfast, Northern Ireland

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Ageing studies have identified two cytokine promoter polymorphisms that have shown repeated associations IL-6 (-174) and IL-10 (-1082). Others have failed to confirm these associations. This is due in part to studies of limited sample sizes examining a restricted number of cytokine polymorphisms. The inflammatory processes that characterize the cell death that is the hallmark of neurodegenerative disorders such as Parkinson's disease, may also be influenced by cytokine polymorphisms. However as with ageing, the results to date have been inconsistent although a number of studies have suggested the IL-1β (-511) and TNF-α (-308) show significant association with Parkinson's disease susceptibility.

Given the complexity of the cytokine network, and the dynamic interplay between anti and proinflammatory aspects, cross-sectional studies examining many cytokine variants in large sample series are now warranted. Genome-wide association studies may hold promise in resolving the role of cytokine polymorphisms in the inflammatory processes in both disease and ageing.

1 Introduction

Ageing is a complex, multifactorial process which can be defined as a progressive, generalized impairment of function resulting in a loss of adaptive response to stress and an increasing risk of age-associated disease [44, 45, 51–53]. Although the specific biological basis of ageing remains obscure, molecular investigations over the last 50 years have led to a cluster of theories that attempt to resolve ageing across species. However the complexity of the ageing process has only been reflected in the numerous hypotheses that have been proposed [72].

There is increasing evidence from a growing number of studies that longevity has heritability in families. However, a familial history of longevity could also be caused by a shared environment. A study of Danish twins noted only modest heritability in the ability to reach the septuagenarian years and above; no evidence for an effect of shared family environment was found [47]. Other studies show that siblings of centenarians are shown to have a 4-fold higher survival rate to ages above 85 years compared to siblings of persons who died at the age of <75 years [89, 90]. Whereas the twin study examined correlations of age at death in those of old age, the latter study focused on those who survive to extreme old age, and was therefore more likely to detect a stronger effect if genetic factors play a role in longevity.

As the world of scientific research moves into a new era beginning with the publication of the human genome and the continual description of novel variations in genes, the prospect of greater resolution to the question of ageing is at hand. The draft publication of the human genome was released on February 16th 2001, on the day when one of the pioneers of modern science, Charles Darwin, would have reached the ripe old age of 192 years. The human genome is composed of 46 chromosomes that are estimated to encode for between twenty and thirty thousand genes (approximately 40 % of which are functionally unresolved) [56, 117]. Extensive variation has also been observed at the nucleotide level. DNA sequence variants are

estimated to occur in one in every three to five hundred base pairs and are linked with a large diversity of phenotypes, ranging from variation in traits e.g., eye colour, height, susceptibility to disease and even the variation in the rate of ageing.

Polymorphism, literally translated "multiple forms", is the term used to describe the DNA variants that exist within a species. Polymorphism in a gene may result in increased/reduced protein production or affect the level of the abnormal proteins generated, through directly influencing gene transcription [115]. There is increasing evidence to show that gene expression is regulated by complex interplay, from simple polymorphic variants in regulatory regions such as the promoter or 3' untranslated regions, to the presence of microRNA species that can work through a negative/positive feedback mechanism.

At the recent 55th American Society of Human Genetics (2005) meeting held in Salt Lake City, the International HapMap project released its Phase I data. This project's major aim was to help identify linkage disequilibrium patterns traversing the entire human genome and facilitate the identification of haplotype "tagging" single nucleotide polymorphisms (SNPs) [2, 22]. In 2006, public databases (such as dbSNP) housed data on more than nine million candidate human SNPs for which genotype data is available for nearly two and a half million of them [2]. These vast volumes of data, coupled with the incredible advances in genotyping and DNA sequencing technologies, create a situation whereby the geneticist can pinpoint a trait loci [68, 114]. Genome-wide association studies are being proclaimed as the latest, and perhaps most powerful, tool in the mapping of causative/modifying genetic loci in complex disorders such as ageing [101].

The human genome displays a considerable level of inter-individual variability from simple SNPs and short repeats to large-scale deletions, multiplications and rearrangements. Recent studies have demonstrated that large gene copy number variations (CNVs) occur frequently in the general population with for the most part no determinable disadvantage to carriers. However this phenomenon can be pathogenic and in rare cases result in severe disease phenotypes [99, 111, 121]. The severe disease state is usually caused through either a "gain or loss of function" that occurs from an altered balance in the level of the essential protein. However, the presence of variants that give rise to much milder phenotypes and produce a fractional increase/decrease in gene expression may result in the slow, progressive manner of symptoms that typify both the ageing process itself and also the numerous age-related diseases, including cardiovascular disease, cancer and neurodegenerative disorders.

Human longevity appears to be inextricably linked with optimal functioning of the immune system, suggesting that specific genetic determinants may reside in polymorphic loci in genes that regulate immune response. The deterioration of the immune system due to "immunosenescence" (age-associated immune deficiency), coupled with the associated increase in the susceptibility to infectious disease, cancer and autoimmune disorders has restricted the potential human lifespan [14, 32, 34]. However this deterioration of the immune function accompanied by an increased risk of morbidity and mortality observed in the noncentenarian elderly is in sharp contrast to the more intact immune function of centenarians [33]. Profound and complex changes within the humoral, cellular, and innate immune responses occur during the ageing

process, therefore immunosenescence is reflected in the sum of dysregulations of the immune system and its interactions with the other major systems in the human body.

Cytokines are proteins that have a key function, as intercellular messengers, during immune responses and in tissue remodelling. The cytokine network plays a pivotal role in the regulation of the specific type and magnitude of immune and inflammatory response. Consistent with the "remodelling theory" of immunosenescence, differing levels of cytokine production are reported in the elderly and centenarians [8, 9, 96–98]. A potent inflammatory response is vital in the defense against pathogens throughout life and may positively influence reproductive success, but chronic inflammation appears to be a common component in the development of major age-related diseases. This "trade-off" effect is largely predictable since advanced age does not seem to have been foreseen by evolution [14]. Many age-related diseases display altered cytokine profiles, suggesting that an inflammatory pathogenesis may be at the basis of these common causes of morbidity and mortality among elderly. It may be expected that people reaching the extreme limits of human lifespan, having escaped from major-age-related diseases, i.e. healthy centenarians, will be characterized by having genotypic combinations that produce "optimal" pro/antiinflammatory activity [32].

2 Cytokine Polymorphism in Immunosenescence

In several cytokine genes, polymorphism (mostly SNPs or microsatellites) located within the critical promoter or other regulatory regions, is reported to affect gene transcription resulting in inter-individual variation in levels of cytokine production (Table 1). Any qualitative or quantitative effect on cytokine production will ineluctably impinge upon the synthesis and secretion of effector molecules downstream in the cytokine cascade and may therefore alter the immune response. The polymorphic nature of the cytokine genes may confer flexibility on the immune response with certain alleles promoting differential production of cytokines. These then may

Table 1 Position and polymorphisms in selected cytokine genes

Cytokine	Nucleotide position	Polymorphism
IL-1α	-889	C/T
IL-1β	-551	C/T
	+3953	C/T
IL-2	-330	T/G
IL-6	-174	C/G
IL-8	-251	A/T
IL-10	-1082	G/A
IFN-γ	Intron 1	(CA)n
	+874	A/T
TNF-α	-308	G/A
TNF-β	+252	A/G
TGF-β	-800	G/A
	-509	C/T
	+869	T/C
	+915	G/C

Table 2 Positive studies on cytokine gene polymorphisms in young, elderly and centenarians

Study	Gene polymorphism	Centenarians	Elderly (age)	Young (age)	Population	Results
Bonafe et al. (2001)	IL-6 –174 C/G	68 M	150M(60-99)		Italian	↓ GG
Rea et al. (2003)	IL-6 –174 C/G		58 M(80-97)	75 M (19-45)	Irish	↓ GG
Christiansen et al. (2004)	IL-6 –174 C/G	178	1058 (60-95)	474 (18-59)	Danish	↑ GG
Lio et al. (2002)	IL-10 –1082A/G	31 M		161 M (18-60)	Italian	↑ GG
Lio et al. (2003)	IL-10 –1082A/G	72 M		115 M (22-60)	Italian	↑ GG
Lio et al. (2004)	IL-10 –1082A/G	54 M		110 M (18-60)	Italian	↑ GG
	IFN-γ +847T/A	142 F		90 F (19-45)	Italian	↑ A

↑ and ↓ represent a statistically significant ($p < 0.05$) increase or decrease of alleles or genotypes respect to control population.
M = male; F = female.

influence the outcome of viral and bacterial infections and/or increase susceptibility/resistance to autoimmune disorders. In summary, cytokines are essential in all areas of the immune response, so any age-related variation may be of crucial importance in determining whether intact immune function remains preserved. Herein we examine the current literature available investigating the frequency of cytokine polymorphism in ageing (Table 2 and 3).

2.1 Tumor Necrosis Factor Gene Cluster

The human leukocyte antigen (HLA) has been described as a gene system that regulates both the immune system and the ageing process. The HLA plays a central role in antigen presentation and immunosurveillence, and a number of studies have been carried out to investigate whether there is evidence of polymorphic association with immunosensecence [15, 16]. Studies have shown that the HLA-A1, B8, DR3 ancestral haplotype (8.1 AH) is associated with a variety of immune dysfunctions, autoimmune diseases and displays gender specific longevity association [15, 86]. Also of interest, the 8.1 AH is associated with variant immune responses and altered cytokine secretion patterns [15]. This HLA haplotype is also associated with a genetically-determined, high production setting for tumor necrosis factor-α (TNF-α) [60].

The *TNF* gene locus is found within the central HLA Class III region and determines the strength, effectiveness and duration of local and systemic inflammatory responses, as well as repair and recovery from infectious and toxic agents. *TNF* genes show strong linkage disequilibrium with HLA Class I and II genes, and with other genes in the HLA region that are factors in immunoregulation [58]. The multiple pro and antiinflammatory activities of TNF-α and related cytokines in the *TNF*

Table 3 Selected negative studies on cytokine gene polymorphisms in young, elderly and centenarians

Study	Gene polymorphism	Population	Centenarians	Elderly (age range)	Young (age range)
Wang et al. 2001	IL-1α -889 C/T	Finnish		52 M (90)	400 (18-60)
	IL-1α -889 C/T	Finnish		198 F (90)	
Cavallone et al. 2003	IL-1α -889C/T	Italian	40 M	160M(65-99)	478 M (19-65)
	IL-1α -889C/T	Italian	94 F	149F(65-99)	210 F (19-65)
	IL-1β -511C/T	Italian	40 M	160M(65-99)	478 M (19-65)
	IL-1β -511C/T	Italian	94 F	149F(65-99)	210 F (19-65)
Wang et al. 2001	IL-1β -511 C/T	Finnish		52 M (90)	400 (18-60)
	IL-1β -511 C/T	Finnish		198 F (90)	400 (18-60)
	IL-1β +3953	Finnish		52 M (90)	400 (18-60)
	IL-1β +3953	Finnish		198 F (90)	
	IL-1raVNTR86bp	Finnish		52 M (90)	
	IL-1raVNTR86bp	Finnish		198 F (90)	
Cavallone et al. 2003	IL-1raVNTR86bp	Italian	40 M	160M(65-99)	478 M (19-65)
	IL-1raVNTR86bp	Italian	94 F	149F(65-99)	210 F (19-65)
Ross et al. 2003	IL-2 – 330 T/G	Irish		28M (80-97)	41 M (19-45)
	IL-2 - 330 T/G	Irish		65 F(80-97)	59 F (19-45)
Scola et al. 2005	IL-2 - 330 T/G	Italian	168		214
Wang et al. 2001	IL-6 –174 C/G	Finnish		52 M (90)	400 (18-60)
	IL-6 –174 C/G	Finnish		198 F (90)	
Bonafe et al. 2004	IL-6 –174 C/G	Italian	255 F	227F(60-99)	
Ross et al. 2003	IL-6 –174 C/G	Irish		55 M(80-97)	69 M (19-45)
	IL-6 –174 C/G	Irish		127F(80-97)	120 F (19-45)
	IL-6 –174 C/G	Irish		135F(80-97)	107 F (19-45)
Capurso et al. 2004	IL-6 –174 C/G	Italian	19 M		44 M (19-73)
	IL-6 –174 C/G	Italian	62 F		78 F (18-73)
Pes et al. 2004	IL-6 –174 C/G	Italian	36 M		68 M (60)
	IL-6 –174 C/G	Italian	76 F		68 F (60)
Ross et al. 2003	IL-8 -251 A/T	Irish		28 M(80-97)	41M (19-45)
	IL-8-251 A/T	Irish		65 F(80-97)	59 F (19-45)
	IL-10 –1082A/G	Irish		28 M(80-97)	41 M (19-45)
	IL-10 –1082A/G	Irish		65 F(80-97)	59 F (19-45)
Wang et al. 2001	IL-10 –1082A/G	Finnish		52 M (90)	400 (18-60)
	IL-10 –1082A/G	Finnish		198 F (90)	
Lio et al. 2002	IL-10 –1082A/G	Italian	159 F		99 F (18-60)
Lio et al. 2003	IL-10 –1082A/G	Italian	102 F		112 F (22-60)
Pes et al. 2004	IL-10 –1082A/G	Italian	32 M		31 M (60)
	IL-10 –1082A/G	Italian	55 F		54 F (60)
Ross et al. 2003	IL-12 exon8 A/C	Irish		28 M(80-97)	41 M (19-45)
	IL-12 exon8 A/C	Irish		65 F(80-97)	59 F (19-45)
	IFN-γ intron 1	Irish		28 M(80-97)	41 M (19-45)
	IFN-γ intron 1	Irish		65 F(80-97)	59 F (19-45)
Lio et al. 2002	IFN-γ +847T/A	Italian	32 M		158 M (19-45)
Pes et al. 2004	IFN-γ +847T/A	Italian	32 M		36 M (19-45)
	IFN-γ +847T/A	Italian	64 F		58 F (19-45)
Wang et al. 2001	TNF-α -308G/A	Finnish		52 M (90)	400 (18-60)
	TNF-α -308G/A	Finnish		198 F (90)	
Lio et al. 2003	TNF-α -308G/A	Italian	72 M		115 M (18-60)
	TNF-α -308G/A	Italian	102 F		112 F (18-60)

Table 3 (continued)

Study	Gene polymorphism	Population	Centenarians	Elderly (age range)	Young (age range)
Ross et al. 2003	TNF-α -308G/A	Irish		28 M(80-97)	41 M (19-45)
	TNF-α -308G/A	Irish		65 F(80-97)	59 F (19-45)
	TNF-β +252A/G	Irish		28 M(80-97)	41 M (19-45)
	TNF-β +252A/G	Irish		65 F(80-97)	59 F (19-45)
Carrieri et al. 2004	TGF-β1 –800G/A	Italian	50 M		94 M (20-60)
	TGF-β1 -800G/A	Italian	122 F		153 F (20-60)
	TGF-β1 -509C/T	Italian	50 M		94 M (20-60)
	TGF-β1 -509C/T	Italian	122 F		153 F (20-60)
	TGF-β1 +869C/G	Italian	50 M		94 M (20-60)
	TGF-β1 +869C/G	Italian	122 F		153 F (20-60)
	TGF-β1 +915C/G	Italian	50 M		94 M (20-60)
	TGF-β1 +915C/G	Italian	122 F		153 F (20-60)

cluster make them attractive candidates along with other HLA genes for unravelling the molecular mechanism(s) underlying the development of ageing and related diseases.

The *TNF* cluster, containing both *TNF*-α and *TNF*-β genes, has numerous polymorphisms that may have an effect on transcription and ultimately protein levels. The *TNF*-α-308A/G polymorphism has been investigated in four studies that were aimed to assess its association, if any, with longevity. In studies of elderly and young subjects from Finland and Sweden there were no differences regarding the frequency of the *TNF*-α-308A/G polymorphism between the two age groups [19, 119]. In an Italian study, the frequency of this polymorphism did not vary between centenarians and younger subjects, and no significant gender difference emerged [63]. Likewise, the frequency of *TNF*-α -308A/G polymorphism in a study with Irish nonagenarians was not different compared to younger controls [105]. In the same study, no significant frequency difference for the *TNF*-β +252 A/G polymorphism between Irish nonagenarians and young control subjects was found either. Thus, these 4 studies appear to demonstrate that the *TNF*-α -308A/G polymorphism does not have a major independent effect on longevity.

2.2 Transforming Growth Factor-β

The cytokine transforming growth factor-β (TGF-β) has been shown to have an essential role in inflammation and in maintenance of immune response homeostasis. TGF-β belongs to the group of cytokines with antiinflammatory effects, due to its deactivating properties regarding macrophages [57, 120]. *TGF*-gene overexpression has been observed in human fibroblasts that displayed a senescent-like phenotype after exposure to oxidative stress [37]. Polymorphisms in the *TGF*-β gene influencing the cytokine production have been identified and subsequently linked to age-related pathologies, such as Alzheimer's disease [66].

A study by Carrieri et al. (2004) analyzed 419 subjects from Northern and Central Italy, including 172 centenarians and 247 younger controls, to examine the hypothesis that variability of the *TGF*-β gene affects successful aging and longevity [12]. The level of the active cytokine increased with age, and significant differences were found between the age groups for the genotype and allele frequencies at the +915 site but no differences were found for the other tested variants (the -800 G/A, -509 C/T and +869 C/G loci). As this +915 C/G polymorphism results in an arginine to proline substitution at codon 25 within the signal peptide that is cleaved from the TGF-β precursor, it is possible that the substitution could play a role in the efficient production of the mature growth factor or misdirection of the protein. Since TGF-β is immunosuppressive, the age-related increase of the active cytokine suggests that it could counteract/counterbalance the harmful effects of inflamm-ageing.

2.3 Interleukin-1 Gene Cluster

The interleukin-1 (*IL-1*) gene cluster is located on chromosome 2q13 and is an important mediator of systemic inflammatory responses. Genetic variation of three genes (*IL-1*α, *IL-1*β, and *IL-1Ra*) in the cluster has been investigated in ageing. Three studies to date have examined the frequency of *IL-1* variants in Finnish, Italian and Swedish populations [18, 19, 119]. These studies failed to show any association with either centenarians or elderly individuals in comparison to young controls. Promoter SNPs in this gene cluster are reported to alter gene expression and may therefore give rise to a different expression profile. Also, *IL-1* gene cluster polymorphisms are implicated in a number of age-related pathologies including neurodegenerative disorders such as Alzheimer's and Parkinson's disease [81, 95].

2.4 Interleukin-2

Interleukin-2 (IL-2) cytokine plays a pivotal role in cellular immunity by regulating the activation, differentiation and proliferation of T-lymphocytes during an immune response. In the elderly, decreased levels of proinflammatory IL-2 production and secretion have been reported, leading to a situation of limited T-lymphocyte proliferation and thus inhibited cellular response [13]. A promoter variant of the *IL-2* gene at position -330 T/G has been shown to negatively affect expression levels, demonstrating that the more common T-allele has a higher level of expression. Two studies have examined the frequency of the *IL-2* promoter polymorphism -330G in ageing populations. The first study by Ross et al. did not identify any frequency difference with this SNP and successful ageing in the Irish population [104]. The second study with Italian centenarians supported the lack of association. However, a

trend was observed suggesting that the -330G allele is increased in frequency in the second study's centenarian cohort and thus decreased levels of the proinflammatory IL-2 cytokine promotes successful ageing [109]. Interestingly, although decreased IL-2 production was reported during the ageing process, increased levels of interleukin-6 (IL-6) were found [78].

2.5 Interleukin-6

The interleukin-6 (IL-6) protein is arguably the best cytokine candidate to act as a potential marker for the overall health status of an individual [27, 67]. This is partly due to the fact that IL-6 plays a major role in inflammation and in the humoral immune response. It has been reported that healthy elderly individuals and centenarians exhibit a proinflammatory status, with a distinctive increase in IL-6 production [32]. There is increasing evidence for directly proportional association of the *IL-6* -174 C/G promoter polymorphism (Fig. 1) with production levels of the cytokine and with age [7, 30, 85].

Bonafe and colleagues (2001) published that the homozygous -174GG genotype was a disadvantage for longevity in men, reporting associated higher IL-6 serum levels. The findings of Ross et al. that an overall decrease in the -174GG in a cohort of Irish octo/nonagenarians from the Belfast Elderly Longitudinal Free-living Ageing Study (BELFAST) study group and with particular respect to the total males in comparison to the controls during the study would appear to concur with the findings by Bonafe et al. However, Olivieri and colleagues (2002) reported that their subjects containing the -174C allele showed a significant age-related increase in the capability to produce IL-6, even though this genotype is supposed to predispose these individuals to be low producers [85]. Another study by Wang et al. in Finland detected no significant change in *IL-6* frequencies

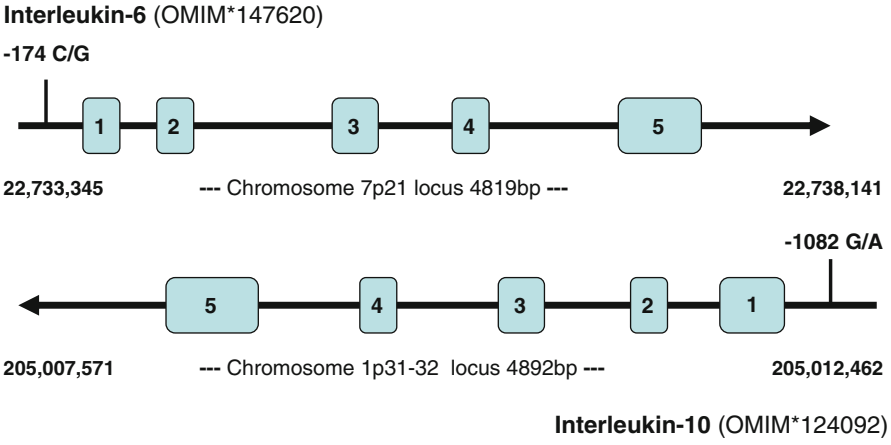

Fig. 1 Genomic structures of IL-6 and IL-10

between nonagenarians and blood donors, though a reduction of 2% was noted in –174GG frequency in comparison with their age-selected younger control group [119]. This trend for a reduction in –174GG homozygosity in elderly males in 3 countries across Europe seems intriguing since it appears to confirm in different study populations and with alternate study designs like the earlier findings obtained in the Italian population.

In later studies, there was no difference in the *IL-6* -174 C/G promoter allelic and genotypic frequencies between centenarians and controls, but the number of subjects enrolled in these studies was low [10]. A modest but significant increase in the frequency of *IL-6* –174 GG homozygotes with age was noted in a group of Danish subjects, though no analysis was carried out for gender [20]. This discrepancy may be due to racial/genetic as well as lifestyle and ethnic/cultural differences between these populations.

A total of 9 studies have now looked at *IL-6* polymorphism with respect to ageing [11, 19]. IL-6 looks like the most interesting cytokine with respect to longevity studies, separate Caucasian European elderly populations and with different selection criteria, appear to demonstrate a decrease in the *IL-6*-174GG homozygote frequency with extreme old age. Italian researchers additionally demonstrated a reduction in *IL-6* high producer allele frequency for male centenarians which was not seen in females. In conclusion, large scale studies on many diverse racial and ethnic populations are needed to clarify this important topic.

2.6 Interleukin-8

Interleukin-8 (IL-8) is defined as a “chemokine” due to the observed chemotactic activity for specific types of leukocytes. It is produced by most cell types and is important for the activation of the inflammatory response and acts as a costimulatory factor for T-lymphocyte responses. IL-8 is also a potent neutrophil chemokine that facilitates the movement of neutrophils to inflammatory sites where they limit and contain the infection. As serious infections are more common in the elderly, it has been postulated that aspects of neutrophil function might be comprised with increasing age [23]. Recently, varied levels of IL-8 production by T- and natural killer (NK)-lymphocytes were reported in an elderly Italian population [69]. Only one study to date has examined the frequency of the functional *IL-8* -251 A/T promoter variant in ageing, though the researchers did not identify any association with either allele [104].

2.7 Interleukin-10

Antiinflammatory interleukin-10 (IL-10), which affects both the T- and B-lymphocyte responses, has been reported at an increased level in the elderly [88]. IL-10 is a potent proliferation and differentiation factor for B-lymphocytes and

prevents production of proinflammatory cytokines such as IL-6 and IL-8. Along with significantly increased IL-6 and IL-10 levels, Rink and colleagues (1998) reported significantly increased production of IL-8 by leukocytes from the elderly. In the same paper, significantly decreased levels of IL-2 and interferon-γ (IFN-γ) were also reported [100]. A number of studies have investigated the association of ageing with the *IL-10* -1082 A/G promoter SNP (Fig. 1), and although the studies have not found any evidence for this, three reported an increase of *IL-10* -1082GG homozygous carriers in elderly males of Italian descent [59, 62, 63]. This may highlight a population-specific effect, although it is noteworthy that this association was not confirmed in other European Caucasian populations [91, 104, 119].

2.8 Interleukin-12

NK-lymphocytes play a central role in the innate immune response against bacterial or viral infections and tumors, and the NK-lymphocyte activities are highly regulated by numerous cytokines, particularly interleukin-12 (IL-12). IL-12 is secreted during the earliest stages of infection and inflammatory response, acting as the key immunoregulatory molecule in cellular immune responses. The critical action of IL-12 at the interface between innate and adaptive immune responses means that any age-associated alterations in expression levels are likely to have crucial functional consequences in vivo [96]. Increased levels of total IL-12, due to higher levels of the p40 subunit, are reported in the aged Irish population [96]. In the paper looking at *IL-12* polymorphisms and ageing by Ross et al., similar frequencies of the *IL-12* +16,974 A/C polymorphism in aged versus control subjects were observed. Although there was a trend for AA homozygotes to be underrepresented in elderly males with a 7% decrease in the A allele in elderly males, neither of these decreases achieved significance. Likewise, no apparent difference was present for old and young female subjects.

2.9 Interleukin-18 and Interleukin-19

Interleukin-18 (IL-18) is a proinflammatory cytokine that plays a vital role in both innate and acquired immune response [48]. IL-18 has been shown to induce IFN- γ and is implicated in a number of inflammatory diseases and neurodegenerative disorders [29, 83]. IL-18 serum concentrations are reported to be higher in centenarians compared to the younger population [38]. Frayling and colleagues (2007) reported an association of an *IL-18* SNP (rs5744292) on serum concentrations of the cytokine product and correlated these with physical functionality in the elderly [36]. These findings suggest a possible genetic association of *IL-18* with successful ageing and warrants further investigation.

Interleukin (IL)-19 belongs to the IL-10 family of cytokines, functioning to stimulate the expression of IL-10 (and the gene locus is adjacent to the *IL-10* gene). To date only one study has examined polymorphism of *IL-19* and its association, if any, to ageing. Okayam and colleagues (2007) investigated the frequency of four SNPs in the *IL-19* gene in the Japanese population. The results showed a significant association with age using logistic regression analysis. This preliminary finding requires independent replication to prove its validity.

2.10 Interferon-γ

Interferon-γ synthesis by T- and NK-lymphocytes and play a decisive role in defense against parasitic/viral infections and intracellular pathogens [82]. Proinflammatory IFN-γ was initially recognized for its antiviral activity but has since established itself as a multifunctional cytokine playing an important role in modulating almost all phases of the immune response, particularly the inflammatory response [4].

In centenarians, Lio and colleagues first reported that possession of the IFN-γ +874A allele (+847 SNP T/A is in linkage with a CA repeat microsatellite allele) was associated with longevity in Italian centenarian females likely by controlling inflammatory status [61]. Subsequently, Ross et al. could not be replicate this observation in nearly 200 Irish nonagenarians reporting similar frequencies for the CA 12 allele repeat in control and aged subjects [104]. The small decrease in the CA 12 repeat in aged, Irish, female nonagenarians was not significant but does demonstrate a similar trend to the findings in the Italian centenarian female cohort suggesting a gender-specific effect in *IFN*-γ genotypes. In Ross et al. the CA 13 repeat allele of *IFN*-γ microsatellite was similarly represented between Irish aged and young groups with no gender difference. The study also commented on a notable trend showing a decrease in the frequency of the heterozygote 12, 13 genotype within the aged subjects in comparison to the young controls (which was observed to be independent of gender).

In the aforementioned Italian study, trend of a decrease in the *IFN*-γ + 874T allele was observed among female Sardinian centenarians, however these results relate to only a relatively small number of Sardinian female centenarians, which may limit the statistical power of the study [91]. Thus, the *IFN*-γ high producer haplotype 12 CA/+874T showed a decrease in centenarian Italian females with a trend for the same change in Irish nonagenarians and Sardinian centenarians. These findings certainly warrant further replication studies and gender differences also need to be taken into account.

2.11 Gender Bias

It is postulated that cytokine allele frequencies are possibly gender- and geographically-specific, similarly to what has been proposed for other polymorphic systems

such as the human leukocyte antigen [16]. Franceschi and colleagues (2000) have postulated that gender is a variable concerning the genetics of ageing, proposing that men and women follow different pathways to extreme longevity [35]. This postulate has been demonstrated in Italian centenarian studies for the *IL-6* -174 G/C, *IL-10* -1082 G/A and *IFN*-γ +874 T/A polymorphisms, where significant frequency differences have been identified when the data was analyzed on the basis of gender [7, 62, 85].

The *IL-6* -174GG genotype frequency is reported to be decreased in elderly males in two independent studies in Italian and Irish populations [7, 104]. The *IL-10* -1082 G/A and *IFN*-γ +874 T/A polymorphism have also been reported to be gender-specific markers for longevity [61, 62]. Lio and colleagues (2002) reported an increased frequency of the homozygote *IL-10* –1082GG genotype in Italian centenarian men, and this genotype is associated with high IL-10 cytokine production, conferring an anti-inflammatory status which is postulated to increase the possibility of extreme longevity [62]. The *IFN*-γ +874T allele is reported to be in absolute correlation with the 12 CA repeat allele, where the latter is associated with increased IFN-γ production [61, 93, 94]. The group reported that possession of the *IFN*-γ +874A allele, particularly in females, conferred an overall antiinflammatory status promoting longevity.

2.12 Cytokine Polymorphism Conclusions

Due to the intricate nature of the cytokine cascade and the perpetual interaction of cytokines within the immune function, a situation is created where the overexpression of one cytokine may be either compensated for or enhanced by another. This complexity, coupled with the difficulty of clearly defining cytokine activity as anti or proinflammatory, may well have confounded the groups whose studies are reviewed in this chapter [17]. The aforementioned studies also highlight the importance of validation of significant results in either a second subgroup or independent cohort of subjects. There is also need to identify such variables as race/ethnicity, gender and age when endeavouring to fully ascertain the role(s) of cytokine polymorphisms in immunosenescence.

Genetic variants in immune response genes are certainly attractive candidates to study in the attempt to elucidate the molecular mechanism(s) that occur during immunosenescence. However, the absence of age-association for many of the cytokine gene variants, even those associated with changing expression levels, would indicate the complexity of the cytokine cascade can not be truly reflected by a small number of polymorphic markers. Future studies concentrating on compiling a genetic cytokine profile that encompasses the overall network (and working in tandem with expression levels) will aid in the resolution of the role(s) of cytokines in the aged immune function/longevity.

3 Age-related Disorders

Identification of major genetic variants affecting population mortality and extreme longevity may spur the characterization of pathways high in the hierarchy of the physiological processes that influence the onset of common age-related diseases [46]. Conversely, the study of age-related disease may provide greater insight into the molecular mechanism(s) that contribute to the ageing process. For example, Parkinson's disease (PD) is one of the most prevalent age-related neurodegenerative disorders, with approximately 1% of the population older than 50 years being affected. The question that must be asked is whether age-related disorders such as PD are a direct cause or a result of the ageing process. Unlike the study of progeriod syndromes (which are characterized by accelerated ageing), age-related disorders require more focused attention to particular aspects of the disease that mimic, or contrast with, healthy ageing.

With increasing knowledge of the complexity of the biological pathways of the brain there is growing evidence to suggest that there is an active, endogenous immune system. Glia cells are one of the most numerous cell types in the brain, and a subgroup, microglia, form the tissue macrophage population. The microglia play an important role in the growth and survival of neurons and are also critical in the inflammatory response of the brain through the production and secretion of cytokines [21]. Inflammation of the brain is postulated to contribute to the pathogenesis of a number of neurodegenerative disorders including multiple sclerosis, Alzheimer's disease and PD. The pathological, neuroinflammatory damage that is observed in these diseases has led researchers to generate hypotheses regarding their progression and susceptible neuronal populations. This hallmark neuroinflammation also provides groups with a possible avenue for therapeutic intervention and implicates DNA variants that regulate the inflammatory response in disease pathogenesis. This next section of the chapter will focus on the role of cytokine polymorphisms in PD and how genetic findings in this complex disorder can help guide future studies regarding the mechanisms influencing ageing.

3.1 Parkinson's Disease Background

The renowned French neurologist Jean Martin Charcot (1825–1893) defined the clinical syndrome "maladie de Parkinson" that has since become known as Parkinson's disease. First described by the English physician James Parkinson (1755–1824) in his milestone 1817 publication "An Essay on the Shaking Palsy", parkinsonism is characterized by the triad of tremor, rigidity and bradykinesia. PD is the most common cause of parkinsonism and the second most frequent neurodegenerative disorder, after Alzheimer's disease. Neuropathological findings in PD are loss of pigmented neurons in the brain stem, *substantia nigra* and *locus coeruleus*, with intracellular Lewy body inclusions found within surviving neurons.

Historically PD was thought to have no genetic basis and epidemiological data appeared to support this view. Cross-sectional studies by Tanner et al. and Wirdefeldt et al. suggested that either there is no genetic basis or that it is only evident in early-onset PD (age of onset <50 years), although to date twin studies have been underpowered to refute incompletely penetrant genetic causes of PD [112]. Differing disease concordance rates between monozygotic and dizygotic twins in longitudinal studies (including those using 18F-dopa positron emission tomography; PET) do support heritability in PD [92]. In fact, many clinical reports note that familial aggregation of parkinsonism and a family history of disease is the second most significant risk factor after age [110].

The etiology and pathogenesis of PD remains unclear, however, it has been suggested that PD, like ageing, may be a multifactorial disorder caused by a combination of age, genetic and environmental factors. During the last decade, contention regarding the importance of genetics in PD was challenged by the identification of several large pedigrees in which parkinsonism appeared to have a monogenic, Mendelian pattern of inheritance (either autosomal dominant, autosomal recessive or X-linked) [28, 106]. However, research studies that have analyzed PD families with classical linkage methods have given rise to data which allowed the subsequent nomination of 13 regions of the human genome, where pathogenic mutations since been identified in five genes (α-*synuclein*, *parkin*, *DJ-1, PINK1 and LRRK2*), thus confirming the role of genetics in PD.

The identification of *LRRK2* pathogenic mutations as a cause of autosomal dominant parkinsonism which is clinically indistinguishable from sporadic PD has once again revolutionized this research field. The *LRRK2* c.6055G>A (Gly2019Ser) mutation has become renowned for its high frequency in specific racial groups (e.g., ~40% of PD patients of Berber Arab ethnicity) and appears to be present in most Caucasian populations. The *LRRK2* variant c.7153G>A (Gly2385Arg) may be the most frequent genetic risk factor the development of PD to date, but it appears to be restricted to those individuals of Chinese descent [107]. The reduced penetrance observed for *LRRK2* mutations accounts for the presence of these variants in healthy control subjects and is reflected in the diversity of the age at symptomatic onset of disease in patients. Likewise, even individuals within the same family carrying the same *LRRK2* mutation can present with symptoms decades apart with regards to age. These observations suggest that *LRRK2*-associated disease is regulated by important environmental and/or genetic modifiers [103].

3.2 PD and Cytokines

Evidence has also been accumulating over the last decade to indicate that chronic inflammation of the brain may be one of these possible disease modifiers and play a crucial role in the pathognomic dopaminergic neuronal death of PD [1, 71]. In support of this theory, proinflammatory cytokines, such as TNF-α, IL-2 and IL-6, are shown to be markedly up-regulated in the brain or the cerebrospinal fluid in PD patients [6, 24,

74, 75, 77]. Despite the potentially important role the inflammatory response, directed by cytokines, may play in the pathogenesis of PD, only a limited number of studies have been performed to assess if there is an underlying genetic influence (Table 4).

In 2000, Kruger and colleagues performed one of the first studies investigating the possible role of cytokine polymorphisms regarding susceptibility to and the pathogenesis of PD [55]. This study identified significant associations between two genes in the *TNF* pathway (*TNF*-α and *TNFR1*). These findings implicated the proinflammatory pathway in promoting the dopaminergic neuronal cell death that typifies PD. Between 2000 and 2003 further studies were performed in the Japanese and Finnish PD populations [70, 79–81]. Nishimura and colleagues investigated the frequency of variants in *TNF*-α, *IL-1*ß, chemokine monocyte chemoattractant protein

Table 4 Positive studies on cytokine gene polymorphisms in Parkinson's disease

Study	Population	Gene polymorphism	Patients	Controls	Results
Kruger et al. 2000	German	TNF-α -308 G/A	264 (114F 148M)	198	↑GA
Wahner et al. 2007	US	TNF-α -308 G/A	289 (133F 156M)	269 (130F 139M)	↑ 2^o
Wu et al. 2007	Taiwanese	TNF-α -863 C/A	369 (173F 196M)	326 (143F 183M)	↑AA
Nishimura et al. 2001	Japanese	TNF-α -1031 C/T	172 (103F 69M)	157 (98F 59M)	↑ C
Wu et al. 2007	Taiwanese	TNF-α -1031 C/T	369 (173F 196M)	326 (143F 183M)	↑ CC
Kruger et al. 2000	German	TNFR1 -609 G/T	264 (114F 148M)	198	↓ B/2
Kruger et al. 2000	German	TNFR1 +36 A/G	264 (114F 148M)	198	↓ B/2
Nishimura et al. 2000	Japanese	IL-1β -511 C/T	122	112	↑1^o 1^o (AAO)
Schulte et al. 2002	German	IL-1β -511 C/T	295 (123F 172M)	270 (130F 140M)	↑ T
McGeer et al. 2002	Canadian	IL-1β -511 C/T	100	100	↑ T
Mattila et al. 2002	Finnish	IL-1β -511 C/T*	52 (27F 25M)	73 (34F 39M)	↓ 2^o 2^o
Wahner et al. 2007	US	IL-1β -511 C/T	289 (133F 156M)	269 (130F 139M)	↑ 2^o
Hakansson et al. 2005	Swedish	IL-6 -174 G/C	265	308	↑ GG
Ross et al. 2003	Irish	IL-8 -251 A/T	90 (41F 49M)	93 (65F 28M)	↑ AT
Hakansson et al. 2005	Swedish	IL-10 -1082 G/A	265	308	↑ GG (AAO)
Nishimura et al. 2003	Japanese	MCP-1 -2518 A/G	329 (200F 129M)	340 (190F 150M)	↑ AA (AAO)

↑ and ↓ represent a statistically significant ($p < 0.05$) increase or decrease of alleles or genotypes respect to PD patients.

M =male; F =female. 1^o =allele 1; 2^o=allele 2. AAO= affects age-at-onset of symptoms.

This table highlights the inconsistency in results (*is inversely associted compared to other studies) and nomenclature that is used to describe each cytokine polymorphism.

(*MCP-1*), and chemokine receptor-2 (*CCR-2*) in the Japanese population. Mattila et al. investigated the *IL-1* gene cluster, including *IL-1*α, -ß and *IL-1RN* along with a SNP in the intercellular adhesion molecule 1 (*ICAM1*). The results of both studies supported the hypothesis that genetic variation of proinflammatory cytokine genes may influence PD with SNPs in the *TNF*-α and *IL-1ß* genes associated (Fig. 2). However, a number of the SNPs examined showed no association with PD susceptibility.

A study in the German population also found no evidence of association with the *IL-1*α (-889 C/T) SNP with risk of PD [76]. Ross and colleagues (2004) investigated a cross-section of promoter variants in proinflammatory cytokine genes *IL-2* (-330 T/G), *IL-6* (-174 C/G), *IL-8* (-251 A/T) and *TNF*-α (-308 G/A) [108]. Although no association was observed for the variants of *IL-2*, *IL-6* and *TNF*-α, a significant decrease in the number of TT homozygous carriers for the *IL-8* gene was observed in the PD patients. Interestingly, IL-8 is also known as CXCL8 and belongs to the specific group of cytokines known as chemokines.

The cells of the brain, particularly neurons, are believed to possess a wide range of chemokine receptors [73]. Neurological injury and PD are often associated with the increase of nitric oxide and free radicals from glial cells in the brain [64, 113]. At sites of inflammation, brain cells are exposed to high concentrations of reactive oxygen species and reactive nitrogen intermediates (produced by activated neutrophils, macrophages and T-cells) as a normal part of the immune response. A potential variation in IL-8 expression in PD subjects may facilitate the influx of neutrophils, immune and activated glial cells to sites of damage and inflammation, resulting in increased oxidative damage and cell death. However when Huerta and colleagues (2004) examined the frequency of polymorphic variants in four chemokine genes, *RANTES*, *MCP-1*, *CCR2* and *CCR5*, no significant associations with PD were observed in the Spanish population studied [49]. These results support earlier results for *MCP-1* and *CCR2* observed by Nishimura et al. in the Japanese population.

In two Swedish studies by Hakansson and colleagues the frequency of several cytokine polymorphisms were studied [42]. No association with PD was observed for variants in the *IFN*-γ, *IFN*-γ*R2*, platelet-activating factor acetylhydrolase and

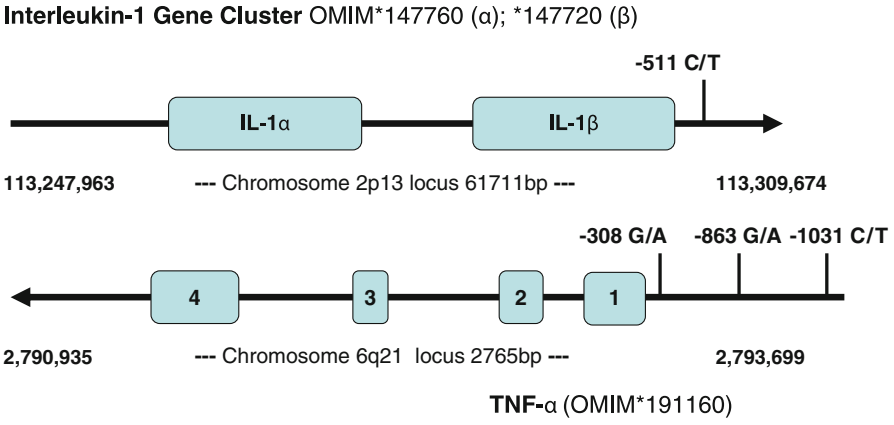

Fig. 2 Genomic structures of IL-1cluster and TNF-α

ICAM1 genes. Likewise, a promoter SNP in the *IL-10* gene did not demonstrate any association with susceptibility to PD, however it did appear to affect the age-at-onset with a significantly higher frequency of the A-allele in early-onset PD patients. Interestingly, the *IL-6* (-174 G/C) promoter SNP did show association with PD with an increased number of -174 GG carriers in the PD patients. The authors further suggest this association is stronger when interactions with a SNP in the *estrogen receptor*-β gene were considered [41].

The latest two papers on this topic have looked at the primary genes that were implicated in PD pathogenesis, *TNF*-α and *IL-1*β [118, 122]. Wu et al. (2007) investigated 4 -promoter SNPs of the *TNF*-α gene (-308, -857, -863 and -1031) in their Taiwanese samples and identified a significant association for the -863 and -1031 SNPs, which were found to be in high linkage disequilibrium in the study. The strongest association was observed with an increased frequency of the -1031 CC genotype in PD patients, and these results concur with the earlier Japanese study by Nishimura et al. (2001) where an increase in this allele with early-onset PD was observed. Wahner et al. (2007) examined the *TNF*-α (-308 G/A) and the *IL-1*β (-511 C/T) SNPs in a US PD patient-control series and observed a significant association with both SNPs, suggesting each SNP individually increased the risk of PD by two-fold and when combined three-fold (Fig. 2).

3.3 Age-Related Disorders Conclusions

It is possible that cytokine polymorphism and genetic variants influence susceptibility to the development of parkinsonism symptoms. Given this hypothesis, it is then even more likely that these variants will influence pathogenesis of the disease affecting age-at-onset, progression and severity of symptoms. The complex nature of this devastating disease is indicative of a multifactorial disorder that is influenced by environmental agents (e.g., infection) acting on a genomic background of susceptibility. The hypothesis that neuroinflammation enhances the degenerative processes involved in PD implies that anti-inflammatory therapeutics may slow disease progression. The use of nonsteriodal antiinflammatory drugs (NSAIDs) has shown some promise in both PD and Alzheimer's disease [3, 54]. At present the only symptomatic relief comes from dopamine replacement (levodopa) and dopamine agonists, as research into the genetics of PD is still a relatively young field. In conclusion, further studies of cytokine polymorphism and genetic variants within genes of the proinflammatory network in PD are certainly warranted.

4 Perspectives

The first century of this new millennium will bear witness to a new era in both ageing research and clinical practice. We are moving into the postgenomic era and the beginning of individualized medicine and treatment. This holds great promise

for the study of complex disorders such as ageing. The field of longevity genetics ("Longevics") will mature, and the identification of mutations that affect the immune system and prolong life will once again revolutionize our views of the ageing process [102]. A strong immune response is clearly important for survival, however this double-edged sword can also increase morbidity. The studies described in this chapter on cytokine gene polymorphisms and ageing certainly suggest that further work is warranted in order to achieve a greater understanding of the complex mechanism(s) underlying longevity.

The intricate nature of the cytokine cascade suggests that any imbalance may be detrimental to the individual. This possible imbalance may be due to an aberrant genomic background of the cytokine network and may be exaggerated by external forces such as infection, stress, smoking and/or diet. Many researchers support the pleiotrophic effect hypothesis as to why immunosenescence occurs by the immune system/response. To elaborate on this concept, it is thought that a proinflammatory genome-genotype ("genomotype") is beneficial in early-childhood and development when the individual is prone to infection. However as we survive past our optimal age and generalized degeneration begins, the proinflammatory genomotype becomes detrimental thus causing damage and promoting autoimmune disorders. Therefore what this phenomenon suggests is that each individual's inflammatory genomotype may behave either as a positive or negative influence on lifespan and this outcome is ultimately determined by the individual's specific environment.

By necessity, both the immune response and the ageing process are determined by genetic, environmental and stochastic factors. Each component is thought to produce a different size effect on a single biological pathway and therefore comparisons are extremely difficult to make. Therefore, the identification of genetic and environmental factors involved in regulating the cytokine response will help highlight other biological pathways that are important in maintaining a healthy immune system. A further confounding effect is gender, as a consistent increased number of females are becoming centenarians than males. The reason(s) for this difference remain unresolved, although studies suggest that gender may effect a differential immune responsiveness which then leads to an inflammatory phenotype. This observation of a gender-bias is supported by similar findings in age-related diseases such as PD, which is more prevalent in males.

Given the complexity previously described, how can one objectively measure the contribution of cytokine genetics to immunosenescence? This question is of course applicable to every pathway postulated to have an effect on the ageing process. Likewise, the underlying answer may also be the same being that one must measure the genetic influence(s) of the overall pathways. To date, as reviewed in this chapter, most studies have examined a small number of variants in one or two cytokine genes and observed inconsistent associations. This has in part been due to small sample sizes and financial restraints. However, the advances that are being made in molecular genetic techniques (including large-scale, rapid genotyping and direct DNA sequencing) will allow objective measurements of cytokine genetics with respect to immunosenescence to be ascertained. This means there is an unprecedented opportunity now available to help unravel the mechanism(s) of the immune system regarding ageing and age-related disorders.

Genome-wide association studies provide an objective measure of genetic variations and are an extension of the classical patient-control studies. These studies are now common-place in leading scientific journals (Nature, Science and the New England Journal of Medicine). Recent genome-wide association studies have identified polymorphisms of cytokine genes as being involved in numerous age-related disorders, like multiple sclerosis [5, 25, 26, 39, 40, 43, 65, 84, 116]. It remains to be seen if these types of association studies will lead to the identification of those genetic factors which influence ageing. These studies will likely require a large collaborative effort to obtain the necessary numbers of aged individuals that will provide the statistical power needed to observe moderate "effect sizes". The Genetics of Healthy Ageing (GEHA) is a current European study of nonagenarian sibling pairs which should have the statistical power to elucidate whether the earlier suggestive changes in cytokine polymorphisms in relation to age and perhaps gender have any coherence, across heterogeneous populations of nonagenarian siblings [31].

Ageing research is becoming of greater interest and importance. The average human lifespan continues to be increased, which has resulted in an expanding proportion of elderly people in society. The developments in molecular, gerontological research have created the potential for survival beyond that of centenarians. The development of stem cell research alone could allow for the generation of completely new cells and organs. In the case of PD, mouse embryonic stem (ES) cells have been used for cell replacement therapy in an animal model of PD [50, 87]. From cultured ES cells, Kim and colleagues (2002) were able to generate a supply of neurons that produce dopamine. The neurons functioned normally and gave clear behavioural responses when grafted into the brains of rats that model PD. The potential benefits of revolutionary experiments like this regarding the treatment and possible prevention of age-related pathologies and perhaps even the rate of ageing itself mean that the deteriorative processes of ageing may no longer be the scourge of mankind.

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Role of TLR Polymorphisms in Immunosenescence

Carmela Rita Balistreri, Giuseppina Candore, Giuseppina Colonna-Romano, Maria Paola Grimaldi, Domenico Lio, Florinda Listì, Sonya Vasto, Letizia Scola and Calogero Caruso

Contents

Abstract: Innate immunity provides a first line of host defense against infection through microbial recognition and killing while simultaneously activating a clonotypic immune response. Toll-like receptors (TLRs) are principal mediators of rapid microbial recognition and function mainly by detection of pathogen-associated molecular patterns (PAMPs) that do not exist in the host. The different members of TLRs recognize several PAMPs, such as peptidoglycan for TLR2, lipopolysaccharide (LPS) for TLR4, flagellin for TLR5, and CpGDNA-repeats for TLR9. Several endogenous ligands of various TLRs have been also identified in the host. In this chapter, we describe the involvement of TLR-4 polymorphisms in immunosenescence, and in particular in age-related diseases, suggesting the crucial role of molecules of innate immunity on these diseases pathophysiology. Hence, we observed that proinflammatory alleles may be related to unsuccessful aging as atherosclerosis and Alzheimer's disease; reciprocally, controlling inflammatory status by antiinflammatory alleles may allow to better attain successful aging.

Keywords: Alzheimer's disease • Atherosclerosis • Longevity • TLR4

C. R. Balistreri (✉) · G. Candore · G. Colonna-Romano · M. P. Grimaldi · D. Lio · F. Listì · S. Vasto · L. Scola · C. Caruso
Gruppo di Studio sull'Immunosenescenza
Dipartimento di Biopatologia e Metodologie Biomediche
Università di Palermo, Corso Tukory 211
90134 Palermo, Italy
Tel.: +390916555911
Fax: +390916555933
E-mail: marcoc@unipa.it or crbalistreri@unipa.it

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1 Introduction

Ageing and longevity are due to a complex interaction of genetic, epigenetic and environmental factors [1]. The genetic component seems to have a relevant role in the attainment of longevity, because it is involved in cell maintenance systems, including immune system. An optimal performance of the both innate and clonotypic branches of immune system has been correlated with survival to extreme ages [2]. The ageing of immune system, known as immunosenescence, is the consequence of changes of clonotypic and innate immune cells caused by lymphoid tissue involution and chronic antigenic overload. The antigenic stress affects the immune system thorough out life with a progressive activation and generation of inflammatory responses involved in the pathophysiology of age-related diseases. Most of the parameters influencing immunosenescence appear to be under genetic control, and immunosenescence fits with the basic assumptions of evolutionary theories of aging, such as antagonistic pleiotropy. Accordingly, the innate immune system, by neutralizing infectious agents, plays a beneficial role until the time of reproduction and parental care, but, by determining a chronic inflammation, can play a detrimental one late in life, in a period largely not foreseen by evolution. In contrast, the clonotypic immune system, with advancing age, shows an exhaustion, due to accumulation of memory cells, which fill the immunological space [2]. As already mentioned, the genetic background seems to modulate the functionality of innate/inflammatory and clonotypic responses and consequently the inflammatory state occurring with advancing age [1, 2]. So, genes encoding molecules involved in innate/clonotypic immunity might influence the susceptibility to age-related diseases and the survival to extreme ages. In other words, the presence of pro/antiinflammatory genotypes might determine a negative or positive control of inflammation, influencing the susceptibility to age-related diseases and/or promoting longevity [2].

In this chapter, we describe the involvement of Toll-like receptor (TLR) 4 polymorphisms in immunosenescence, and in particular in age-related diseases, suggesting the crucial role of molecules of innate immunity on these diseases pathophysiology. Hence, we observed that proinflammatory alleles may be related to unsuccessful aging as atherosclerosis and Alzheimer's disease (AD); reciprocally, controlling inflammatory status by anti-inflammatory alleles may allow to better attain successful aging.

2 TLR4

The innate immune system is the first line of the defensive mechanisms that protect host from invading microbial pathogens. Host cells express various pattern recognition receptors (PRRs) that sense diverse pathogen-associated molecular patterns (PAMPs), ranging from lipids, lipoproteins, proteins and nucleic acids [3, 4].

Recognition of PAMPs by PRRs activates intracellular signaling pathways that culminate in the induction of inflammatory cytokines, chemokines, interferons (IFNs) and upregulation of co-stimulatory molecules. To date, it has been identified three families of PRRs, usually defined as "the trinity of pathogen sensors": Toll-like receptors (TLRs), NOD-like receptors and RIG-like receptors (RLR). NLRs with known functions detect bacteria, and RLRs are antiviral [3, 4].

TLRs family include, in human beings, 10 members that trigger innate immune responses through nuclear factor-κB (NF-κB)- dependent and IFN-regulatory factor (IRF)-dependent signaling pathways [4, 5]. TLRs are evolutionarily conserved molecules and were originally identified in vertebrates on the basis of their homology with Toll, a molecule that stimulates the production of antimicrobial proteins in *Drosophila melanogaster* [6, 7].

Some molecules of this family are expressed at the cell surface, whereas others are expressed on the membrane of endocytic vesicles or other intracellular organelles (Fig. 1). The structure of these receptors is quaternary and they are composed of an ectodomain of leucine-rich repeats n (LRRs), which are involved directly or through accessory molecules in ligand binding, and a cytoplasmic Toll/interleukin(IL)-1 receptor (TIR) domain that interacts with TIR-domain-containing adaptor molecules (Fig. 2) [8]. The different members of TLRs recognize several PAMPs, such as peptidoglycan for TLR2, lipopolysaccharide (LPS) for TLR4, flagellin for TLR5, and CpGDNA-repeats for TLR9 (Fig. 1) [9–12]. Several endogenous ligands of various TLRs have been also identified in the host [13].

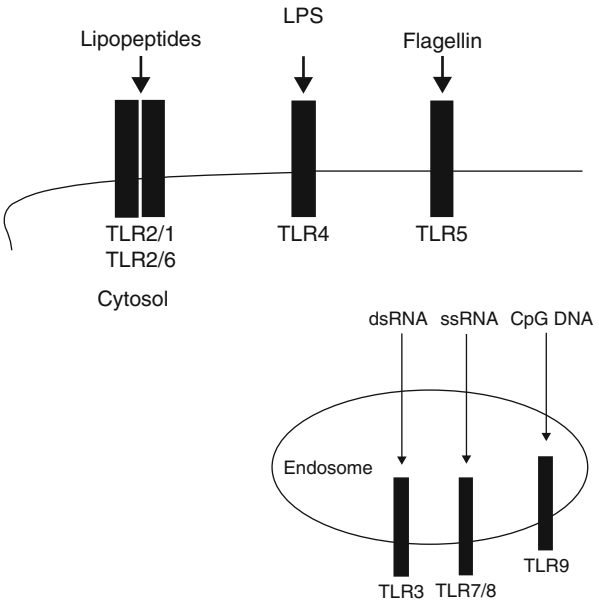

Fig. 1 The members of TLR family are expressed at the cell surface and on the membrane of endocytic vesicles or other intracellular organelles. They recognize several microbial molecules, as shown in the figure.

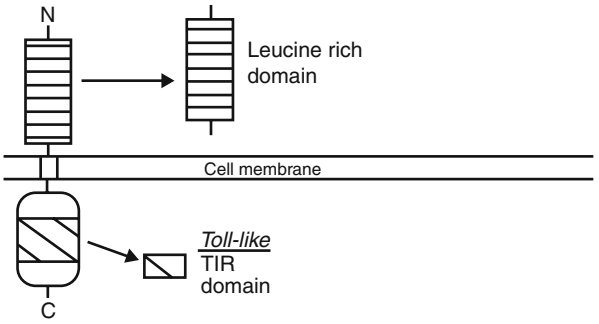

Fig. 2 The TLR structure is composed of an ectodomain of LRRs, which are involved directly or through accessory molecules in ligand binding, and a cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain that interacts with TIR-domain-containing adaptor molecules.

Taking into account their ability to link several molecules, it has been postulated that the genes encoding TLRs receptors would be subject to diversifying selection [14] This is because the proteins are in direct contact with molecules of microbial origin, which might change in structure to evade immune detection. In fact, weak purifying selection seems to apply in the case of the TLRs [14, 15]. Furthermore, the need for detection of various signature molecules seems to come and go in evolution. For example, while some invertebrates are highly sensitive to LPS, most invertebrates are not [16–19]. Drosophila exhibits no response to pure LPS or lipid A at all. Similarly, most vertebrates (fish, amphibians, reptiles, and birds) are at least relatively insensitive to LPS, if not entirely unresponsive [14]. In the case to Danio rerio and Gallus gallus, it is clear that TLR4 encoding genes are represented in the genome. However, in fish and in birds, these TLR4 homologs evidently to not trigger the same set of events as witnessed in mammals. Besides, among mammals, sensitivity to LPS is quite variable, depending upon which endpoint is examined. Humans, anthropoid apes, ungulates, and rabbits are highly sensitive to LPS; mice, rats, and baboons are comparatively resistant [14].

To date, it is possible to suggest that TLRs receptors are the key molecules of natural responses and they also provide a link between innate and clonotypic immunity [20–22]. These evidences have also opened inquiries into previously unknown disease mechanisms [23–25]. Their ability to detect different PAMPs gives a link between infection and various human diseases [23–25]. In fact, members of TLR family have been involved in the pathogenesis of several diseases by studies of people analyzing the incidence of diseases having different polymorphisms in genes encoding TLRs. So, it has been evidenced the crucial role of well-known component of TLR family, the TLR4, in some diseases, as atherosclerosis and AD [24–26].

TLR4 has been identified as the first human homologue of the Drosophila Toll [6, 7]. The extracellular domain of TLR4 that contain over 600 amino acids is highly polymorphic compared with the transmembrane and intracellular domain of the protein [5, 18]. This TLR4 polymorphism contributes to species-specific differences in recognition of LPS, the prototypic TLR4 ligand [5, 19]. The intracellular TIR domain, which is composed of three highly conserved regions, contains

150 amino acids [5, 20]. The TIR domain modulates protein–protein interactions between the TLRs and signal transduction elements [5, 20]. As already mentioned, TLR4 has been shown to be involved in the recognition of LPS, a major cell wall component of Gram negative bacteria [14]. In addition to LPS, TLR4 recognizes several endogenous ligands, such as oxidized-LDL (ox-LDL), lipoteichoic acid, heat-shock proteins (HSP), fibronectin and Aβ amyloid peptide of AD. Its activation by induction of NF-kB and mitogen dependent protein kinase pathways determines the production of cytokines, chemokines, other inflammatory mediators (Fig. 3) [13]. Therefore, it has been suggested that activated TLR4 triggers not only innate immunity but also clonotypic immunity. TLR4 activation on dendritic cells induces the expression of costimulatory molecules and production of inflammatory cytokines [22]. Then, activated dendritic cells present microorganism derived peptide antigens expressed on the cell surface with Major Histocompatibility Complex class II antigen to naive T-cells, thereby initiating an antigen-specific clonotypic immune response [20–22].

TLR4 activity and function may be modulated by genetic polymorphisms (for the most part, single nucleotide polymorphisms, SNPs), prevalently presented in extracellular domain. It has been identified a functional SNP in the human TLR4 gene, an A-G base transition at position +896 base pairs from the transcriptional start site, resulting in an aspartic acid to glycine exchange at position 299 in the amino-acid sequence (referred to as Asp299Gly or +896A/G) [27, 28]. This SNP

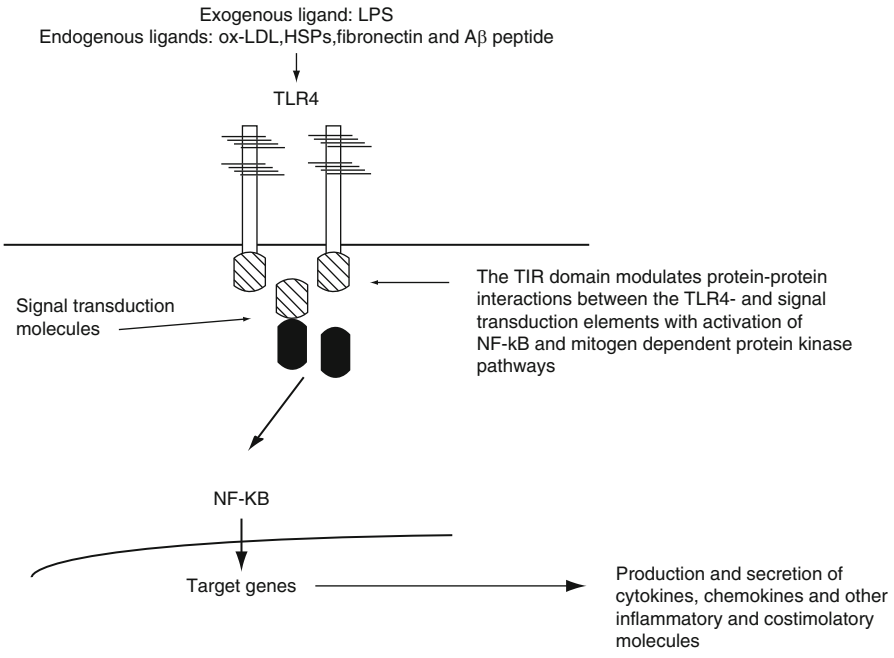

Fig. 3 Activation of TLR4 receptor by LPS (or other agents, as endogenous molecules-as shown in the figure) induces transmembrane signals that activate NF-kB and mitogen dependent protein kinase pathways, determining the expression of a wide number of genes encoding proteins, such as cytokines, with regulatory functions upon leukocyte activation and tissue inflammation.

causes hyporesponsiveness to LPS as well as an increased risk and susceptibility to Gram-negative infections both in human and experimental animals. Recently, it has been suggested that this SNP plays a role in a variety of human diseases, ranging from infectious and inflammatory diseases to cancer [23–26]. So, TLR4 plays a key role in both innate and clonotypic immunity to Gram-negative bacteria and to other agents and it seems to be the hub of inflammatory pathophysiology of age-related diseases, as atherosclerosis and AD [23–26].

3 Involvement of TLR4 in Age-Related Diseases: Its Role in Atherosclerosis, AD, and Cancer

By now, evidence is accumulating that TLR4 could affect atherosclerosis in multiple ways [24, 25, 28–35]. The association between TLR4 and atherosclerosis is consistent with findings showing that TLR4 mRNA and protein are more abundant in atherosclerosis lesions than in unaffected vessels [24, 25, 30]. Furthermore, cultured human vascular endothelial cells express little TLR4 under baseline conditions, and they express high levels of TLR4 on stimulation with proinflammatory cytokines [29]. Among cellular components presented in atherosclerotic plaques are several TLR4-expressing cells, including macrophages, endothelial cells, smooth muscle cells, T-cells and dendritic cells [24, 25, 28–35]. It is largely accepted that ox-LDL as well as other endogeneous ligands, that are expressed during arterial injury, as HSP are responsible for TLR4 ligation and activation. However, taking into account the role of life-long pathogen load on the development of elderly inflammatory status and atherosclerosis, PAMPs should also be involved in TLR4 activation (Fig. 4) [2, 28–37].

To date, there is a large body of genetic data pointing the involvement of Asp299Gly SNP in atherosclerosis development. Ultrasound analysis of carotid arteries in a large Italian population showed that the Asp299Gly was found less frequently in people with progressive lesions representing carotid atherosclerosis, compared with a control group. These results were confirmed by other studies that found a protective effect of the TLR4 variants on acute coronary events. However, other studies investigating a potential association of this SNP with cardiovascular diseases (CVD), as myocardial infarction (MI), did not yield significant results (Table 1) [28, 36–46]. On the other hand, association studies are influenced by a number of possible confounding factors, like the total number of patients and controls and the homogeneity of the population in term of geographical origin among others. Artefacts might occur if the controls are not ethnically matched with the patients.

Literature data have also recently demonstrated the involvement of TLR4 receptor in neurodegeneration. It is now known the role of innate immunity, and precisely of microglial cells, in the inflammatory pathogenesis of AD, as stated by the amyloid cascade/neuroinflammation hypothesis. The former is responsible for the production of the neurotoxic substances, such as reactive oxygen and nitrogen

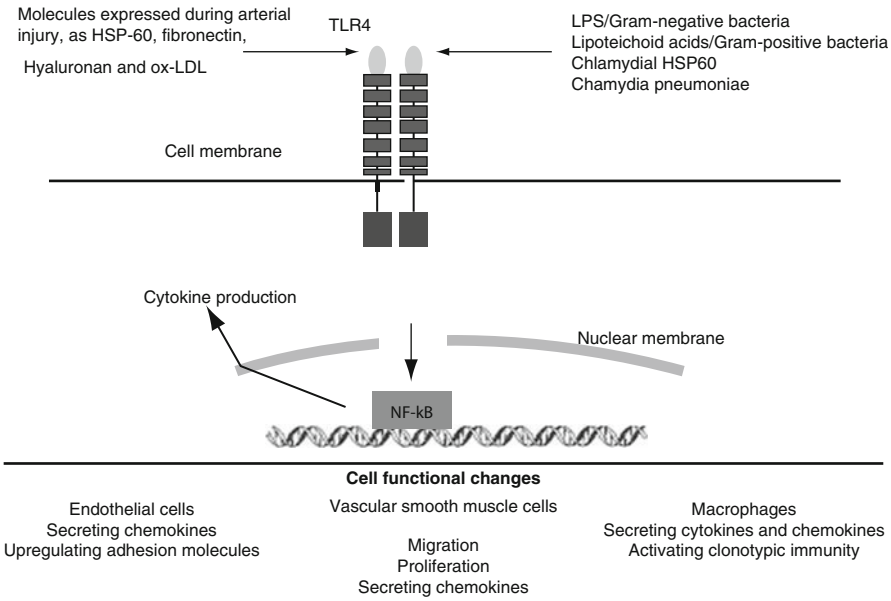

Fig. 4 TLR4 signaling pathway and its relation with atherosclerosis. Both endogenous and exogenous ligands can activate TLR4 on cells, such as endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, dendritic cells and macrophages. Activated TLR4 lead to activation of the NF-KB. This activated transcription factor mediates the expression of several genes and the secretion of proinflammatory cytokines and chemokines, and it also induces expression of adhesion molecules. Ultimately, these processes might initiate or promote atherosclerotic lesions.

species, proinflammatory cytokines, complement proteins and other inflammatory mediators that bring important neurodegenerative changes. Some studies have suggested that activation of microglial cells may be induced throughout the binding of Aβ peptides. Several membrane proteins expressed on microglial cells seem to be implicated in Aβ peptides binding. It has been demonstrated that TLR4 receptor binds highly hydrophobic Aβ peptides aggregates suggesting the production of neurotoxic substances. A further, not mutually, alternative explanation on the key role of microglial activation may be related to the role of TLR4 as LPS receptor. In fact, also in AD life-long pathogen burden has been linked to the pathophysiology of the disease. So it should to be biologically plausible that functional variation in the TLR4 gene might influence the susceptibility to sporadic AD [47–56].

This might be the case for the allelic variants of TLR4 gene, as Asp299Gly SNP, associated, as above described, with an attenuated receptor signaling and a blunted inflammatory response. Association between this polymorphism and AD has been described by Minoretti et al. [57] in an Italian population sample. Our preliminary results of a recent study have confirmed that Asp299Gly polymorphism of TLR4 gene is associated with AD [56].

It has been also suggested the involvement of TLR4 receptor in cancer [23]. It is known the involvement of inflammation, as an etiological factor in several human cancer. Growing evidence suggests that the chronic inflammation induced

Table 1 Summary of studies investigating the potential association of Asp299Gly **TLR4SNP** with cardiovascular diseases

References	Association studies	Results	P-value
Kiechl et al[28]	Carotid stenosis	Participants with SNP have lower incidence of carotid stenosis	0.05
Ameziane et al[38]	Acute coronary events	Participants with SNP have lower incidence of coronary events	0.037
Balistreri et al[39]	MI	Patients (men) with SNP have lower incidence of MI	0.002
Edfeldt et al[40]	MI	Men with SNP have increased incidence of MI	0.004
Zee et al[41]	MI and stroke	Not significant	0.25
Yang et al[42]	Coronary artery stenosis	Participants with SNP have not lower risk of stenosis	0.9
Labrum et al[43]	Carotid events	There was no association between SNP and baseline intima-media thickness (IMT) or progression of IMT over the 3-year follow up	Not significant
O'Halloran et al[44]	Coronary artery disease	There was no evidence overall that the resistance alleles cumulatively influenced the risk of CVD compared to controls or stable angina patients	0.12, and 0.40, respectively
Vainas et al[45]	Peripherical arterial disease	Among patients affected by peripheral arterial disease, TLR4 +896 G allele carriership was univariantly associated with extensive (more than two vascular territories affected) atherosclerotic disease	0.02
Nebel et al[46]	MI	Patients (men) with SNP have not lower incidence of MI	0.36

by different pathogens may also play a role in pathophysiology of some cancer, as gastric cancer [23, 58–62]. Considering that genetic susceptibility is a major risk factor for this disease, it has been hypothesized that sequence variants in genes that regulate inflammatory response may modify individual susceptibility to cancer. So, some studies have analyzed the relationship between the associations of several functional polymorphisms in genes involved in LPS signaling variants and risk of cancer. Garza-Gonzales et al. [59] have investigated the association of Asp299Gly TLR4 SNP and distal gastric cancer in a Mexican population. The results obtained have not demonstrated any association between this SNP and distal gastric cancer, suggesting that it do not contribute to the development to disease. The same data have been obtained in a study performed in a Venezuelan population [60]. In an other study, it has been investigated the role of different SNPs of some inflammatory genes, as Asp299Gly of TLR4 gene, in 377 patients affected by colorectal cancer

and 326 controls from Spain [61]. There was no statistically significant association between this SNP and colorectal cancer risk. However, different results have been found in a study performed in 710 patients affected by lymphoma [62]. In fact, the TLR4 Asp299Gly variant was positively associated with the risk of mucosa-associated lymphoid tissue lymphoma (OR=2.76, 95% CI=1.12–6.81) and Hodgkin's lymphoma (OR=1.80, 95% CI=0.99–3.26). Hence, this study suggests an effect of this SNP in factors of the innate immune response in the etiology of some lymphoma subtypes.

4 Conclusions

Genetic factors play an important role in the ability to achieve exceptional old age, theoretically two class of genes can be considered to be at play [1]. On the one hand, individuals with a genetic make-up useful to achieve extreme old age most likely present with mutations that significantly increase the risk of premature death by lethal, age, and nonage-associated diseases. On the other hand, it has been suggested that genetic variants conferring protection against basic mechanisms of aging and/or age-related illnesses also might exist [1, 2].

To discover the gene factors that let an organism to survive beyond its reproductive age, it is necessary to use an extreme phenotype. From this perspective, the centenarians are the good choice as they represent the survived tail of a very special segment of population. They comprise a cohort of living people who celebrate today the 100th birthday and escaped neonatal mortality, preantibiotic era, fatal outcomes of age-related complex diseases. A small number of centenarians is in quite good heath (in "*good robustness*"), defined as ''group A'' by Franceschi et al., ''escapers'' by Evert et al., and ''exceptionals'' by Gondo et al. [63–65]. The centenarians also represent that segment of population who better adapted and readapted from both biological and non-biological point of view. Centenarians, as representative of longevity, consent to understand the role played by genetic structure of population on the onset of phenotype and the historical dynamism of the longevity trait from a demographic point of view. So, they are the best model for studying the genetics of longevity, and for identifying the genetic factors involved in age-related diseases, since the centenarians represent selected survivors who have clearly delayed or in some cases even escaped age-related diseases, that affect old people and are responsible their morbidity and mortality [66]. Hence, centenarians are a human model of disease-free [67]. In addition, centenarian offspring have increased likelihood of surviving to 100 years and show a reduced prevalence of age-associated diseases, as CVD and less prevalence of cardiovascular risk factors [68, 69]. So, genes involved in CVD may play an opposite role in human longevity, as Asp299Gly SNP. In particular, we have postulated that alleles associated to CVD susceptibility should not be included in the genetic background favoring longevity. So, the genetic background promoting pro-inflammatory responses may play an opposite roles in CVD and in longevity [37, 70–72].

Following a novel approach to study genes involved in CVD and reciprocally in longevity, we have recently demonstrated that antiinflammtory allele of Asp299Gly SNP of TLR4 gene, +896G, is overrepresented in male Sicilian centenarians and underrepresented in men affected by MI, with intermediate values in control population [37]. Thus, our results suggest a role of the innate immune defense system and particularly TLR4 in CVD, and our comparison with the oldest old may help elucidate the role of genetics in age-associated diseases characterized by a multifactorial etiology [39]. Accordingly, TLR4 polymorphisms, which attenuate receptor signaling, enhance the risk of infections, but decrease that of atherogenesis, presumably by limiting inflammatory responses [27, 28]. Hence, the mutation might result in an increased chance of longevity in a modern environment with reduced pathogen load and improved control of severe infections by antibiotics.

However, a recent study has excluded a noteworthy influence of Asp299Gly SNP upon human longevity or MI in German men [46]. The causes of the discrepancies seem be not clear, but the inclusion criteria, the studied populations, and the measured endpoint differed substantially among the studies. Further, it is claimed that results obtained on human populations should always be replicated. Indeed, association with particular genetic polymorphisms and longevity is reported for some population but not for others. However, this is not strange because, as underlined by Capri et al. [73] human populations are characterized by specific gene pools that arise from the particular group's history in terms of chance (genetic drift) and environment (natural selection). Hence, replication cannot reasonably be expected for longevity in light of the considerations discussed in that study.

The suggestion that enhanced male life expectancy is associated with antiinflammatory TLR4 SNP is interesting in view of the role of TLR-4 proinflammatory allele in the control of infectious diseases [25]. In order to rationalize these two seemingly conflicting situations, it might be argued that males carriers of the antiinflammatory allele who are lucky enough not to contact serious bacterial infection earlier in life may have an increased chance of long life survival (trade-off). However the same appears not to be true for female life expectancy [74].

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Part III
Mechanisms - Receptors and Signal Transduction

Signal Transduction Changes in T-cells with Aging

Tamas Fulop, Gilles Dupuis, Carl Fortin and Anis Larbi

Contents

Abstract: There are several functions of T-lymphocytes which are altered with aging. The cause is not exactly known. However the changes in T-lymphocyte activation could be caused by the altered T-cell receptor (TCR) signaling after ligation. The recently described membrane lipid rafts (MR) are critical to the assembly of the TCR, the CD28 coreceptor and the IL-2 receptor signaling machinery. The defect in IL-2 production by $CD4^+$ T-cells with aging is not due to lower levels of expres-

T. Fulop (✉)
Research Center on Aging, Department of Medicine
Immunology Graduate Programme, Faculty of Medicine
University of Sherbrooke, Sherbrooke, Quebec, Canada
E-mail: tamas .fulop@usherbrooke.ca

G. Dupuis
Clinical Research Center, Department of Biochemistry
Immunology Graduate Programme, Faculty of Medicine
University of Sherbrooke, Sherbrooke, Quebec, Canada

C. Fortin
Clinical Research Center, Immunology Graduate Programme, Faculty of Medicine
University of Sherbrooke, Sherbrooke, Quebec, Canada

A. Larbi
Center for Medical Research
Section for Transplant-Immunology and Immuno-Hematology
Tuebingen Aging and Tumor Immunology group
University of Tuebingen, Germany

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sion of the TCR, CD28 or intracellular signaling molecules. However, there is a direct correlation between the activation of $p56^{Lck}$ and LAT at the cellular level and their association/recruitment with the lipid raft fractions of $CD4^+$ and $CD8^+$ T-cells. $p56^{Lck}$, LAT and Akt/PKB are weakly phosphorylated in MR of stimulated $CD4^+$ T-cells of elderly as compared to young donors. Moreover, MR undergo changes in their lipid composition (ganglioside M1, cholesterol) with aging. There exists a differential role for lipid rafts in $CD4^+$ and $CD8^+$ T-cell activation with aging and consequently a differential localization of CD28 which may explain disparities in response to stimulation in human aging, mainly affecting the $CD4^+$ T-lymphocyte population.

Keywords: T-cells • CD4+ T-cells • CD8+ T-cells • Lipid rafts • Signal transduction • Aging Cholesterol • CD28 Coreceptor

1 Introduction

Most of the cell functions occur through specific receptors triggered by various ligands. T-cells possess several receptors which lead to their activation and to the maintenance of their activation status. Antigenic recognition by the T-cell receptor (TCR) triggers a series of biochemical events that result in the expression of a range of genes that are essential to T-cell responses, expansion and effector functions [1]. In addition, ligation of costimulatory CD28, that is required for interleukin-2 production and commitment to proliferation [2], enhances lipid raft polarization [3, 4]. Thus, CD28 triggering is essential for sustained T-cell activation [2]. Once IL-2 is secreted it will act in a paracrine manner on T-cells to trigger their clonal expansion via the IL-2 receptors. Membrane lipid rafts (MR) are dynamic structures and the time-dependent recruitment or exclusion of signaling proteins in these MR control T-cell activation and immune responses [5]. Moreover, lipid rafts are dynamic structures whose composition and function may vary according to cell types and cell subsets, especially in the case of T-lymphocytes [6]. Heterogeneity in MR composition and function may explain disparities in lymphocyte subset functions [7].

2 Signaling via TCR, CD28 and IL-2 Receptors and Their Changes with Aging

2.1 Receptors Involved in T-Lymphocyte Activation

The encounter of pathogens with lymphocytes will initiate their activation resulting in clonal expansion. The cascade of signaling molecules initiated by the stimulation of specific surface receptors results in the activation of several transcription factors. The most important receptors implicated in the clonal expansion of T-cells are the

TCR, the coreceptors including CD28 and the IL-2 cytokine receptor (IL-2R). These receptors function via an intracellular signaling cascade assuring the specificity and the fidelity of the expected response. T-cells need a first signal (signal 1) priming them to the possibility to respond by a clonal expansion to a specific antigen presented in the frame of a major histocompatibility complex (MHC) by an antigen-presenting cell (APC). This will assure that the whole membrane and the early signaling machinery is readily assembled to proceed toward the next stage, that is, the progression toward the full, sustained response. This is ensured by various coreceptors among which the most important is CD28, which represents signal 2. Signals transmitted by this receptor assure that the clonal expansion occurs via a sustained activation by the creation of the immune synapse (IS). Finally, as the concerted CD28 activation leads to the production of IL-2, it should also efficiently stimulate T-cells, representing signal 3. Altogether these receptors act for a complete response of T-cells assuring an adequate response to a specific antigen. With aging several studies have shown that the number of TCR is not changed. The CD28 number, claimed as a biomarker of aging, seems to decrease mainly in the case of a specific T-cell subpopulation, the memory CD8+ T-cells. These cells seem to represent a very late differentiated population characterizing immunosenescence [8]. These cells are the result of continuous chronic stimulations by antigens probably of viral origin including cytomegalovirus (CMV) and other herpes viruses, as similar changes were observed during CMV infection [9]. They are also the result of the low-grade chronic inflammation, however, the inflam-aging theory could not be validated in SENIEUR donors. One naturally arising question is whether this is a normal process related to aging, whether related to age-related disease processes or to the progressing frailty syndrome occurring in certain groups of elderly subjects. Our works suggest that in case of CD4+ T-cells the number of CD28 co-receptor is not decreasing, while it is decreasing in the CD8+ T-cell subpopulation [10]. Whether, the expression of IL-2R change during aging is still controversial, however, our work suggests a maintained expression in healthy elderly individuals. The TCR and CD28 are signaling via two specific cascades, however, there are more and more data suggesting that a cross-talk could exist between these two major pathways. We will describe these signaling pathways individually and in their cross-talk with a special emphasis on changes occurring with aging. However, first we will discuss MR and their role in signaling with special emphasis on the age-related changes.

2.2 Membrane Lipid Raft Function and Composition

One of the most important advances in membrane biology and consequently in the signaling field was the discovery of the existence of lipid rafts in the cell membrane that are now called membrane rafts (MR) [11]. These microdomains are composed mainly of satured lipids, cholesterol, glycosphyngolipids, GPI-anchored proteins, and posttranslationally modified proteins. These high-melting sphingolipids packed with cholesterol generate a liquid-ordered phase (lo) arrangement. This composition forms an efficient signaling platform necessary for an adequate signaling and cell response.

TCR ligation induces a redistribution of phosphorylated proteins into MR, which are highly compact relatively small domains (20–200 nM). The saturation of the lipids as well as the enrichment in cholesterol both allow the rafts to move through the membrane as discrete units. Their movement will be differentially segregated to the various poles of the cell depending on their main specific component, such as ganglioside M1 (GM1), GM3, or flotillin. The consequence of cell polarity is the asymmetric localization of membrane receptors and signaling molecules between the leading edge (at the cell front) and the uropod at the rear edge [12] This cell polarity will also influence the protein composition and the protein–protein interactions into the rafts. Data support the role of MR in the asymmetric distribution of membrane proteins during cell polarization. There is still a debate on which interactions direct and determine the MR movements and functions, and whether they involve cholesterol, membrane proteins, or both [13]. Experimental data seem to indicate that there could be several types of rafts playing different roles [14]. Furthermore, the role of MR is not limited to signal transduction, but also to lipid transport, virus entry, cell movement, as well as cell–cell communication. The accumulation or clusters of signaling molecules via MR initiate the formation of a signaling platform, which increases the efficiency of signaling. T-cell activation is the consequence of the interaction between the TCR and specific antigen presented by the APC. Signal 1 is occurring over a time frame of a few seconds but the interaction between T-cells and APC can be sustained for many hours. This prolonged interaction leads to the formation of the supramolecular activation cluster (SMAC) at the immunological synapse (IS). Thus, the sustained T-cell activation via organized MR signaling ultimately leads to the formation of a mature IS needed to achieve full T-cell activation through the contribution of CD28. The organization and composition of the membrane will directly modulate the formation of such a signaling platform, which ultimately influences cellular activation and functions. Thus, MR play a very important role in signaling by the formation of the signalosome, which are multicomponent transduction complexes. The correlation between the capacity of a molecule to be recruited into the IS and its preference for being linked to membrane rafts is still debated. However, very recent experimental evidence suggest that dynamic rafts reorganization at the IS favor T-cell activation by generating an environment where signal transduction is protected and essentially amplified [12]. Thus, the recruitment and clustering of MR within the IS segregate negative and positive actors of T-cell activation and protect TCR signaling.

Furthermore, the localization of molecules throughout the membrane is dependent on posttranslational modifications including acylation, farnesylation, and palmitoylation. Recently, it was demonstrated that LAT phosphorylation was not optimal in antigen-primed anergic CD4+ T-cells after TCR ligation [15]. It is of interest that LAT association with membrane rafts was defective in these CD4+ T-cells and this was partly explained by the impaired palmitoylation of LAT. It can be supposed that the posttranslational lipidation of the signaling molecules targeting them to MR under stimulation is altered with aging. In T-cells some of the signaling machinery is constitutively included in MR, such as the TCR, Lck, while other molecules are recruited during activation, such as CD28, IL-2R, LAT, PI3K. It is of note that we presented evidence that CD4+ and CD8+ T-cells require differential activation [10].

The signaling machinery in CD4+ T-cells relies on MR for its assembly, while in CD8+ T-cells a certain preassembly of the signalosome decreases the necessity of MR for adequate signaling. This could be perhaps explained by the differential fate of these two T-cell subpopulations. There are still numerous questions to answer on the role of MR in T-cell activation and IS formation, nevertheless, a consensus exists, which states that in a way or another MR participate in T-cell activation.

2.3 The Contribution of Membrane Lipid Rafts to the Altered-T-Lymphocyte Functions with Aging

We have reported an alteration in the function of MR with aging. MR poorly coalesce in $CD4^+$ T-cells of elderly subjects [10] although the alterations are less pronounced in the case of $CD8^+$ T-cells. We have also reported alterations in the recruitment and activation of Lck and LAT into MR of T-cells from aged humans [16]. One important finding was that CD28 and IL-2R were weakly recruited to MR in $CD4^+$T-cells of elderly subjects. In contrast, these proteins were already located to MR in $CD8^+$ T-cells from elderly subjects prior to stimulation. These observations suggested that the assembly of the signaling machinery in $CD4^+$ T-cells relies largely on MR, whereas in $CD8^+$ T-cells a preassembly of the signalosome has been suggested by us [10] and by others [6, 7].

Moreover, MR of $CD4^+$ and $CD8^+$ T-cells behave differently in polarization experiments induced by anti-TCR/CD28-coated beads. While the beads induced MR polarization to the region of contact in $CD4^+$ T-cells of young and elderly individuals, the beads failed to induce coalescence in $CD8^+$ T-cells of both groups of donors. Recently, it was supposed that the expression of CD8 gives to the cell a "dominant-negative" phenotype towards MR polarization [6] as it occurred in immature $CD4^+CD8^+$ T-cells [18]. Thus, MR functions may be settled on during T-cell selection by an unknown mechanism. Altogether these data suggest that $CD4^+$ T-cells heavily rely on MR to reach a full state of activation, while $CD8^+$ T-cells due to their pre-existing signalosome could circumvent "lipid rafting." This raised the possibility that similar age-related changes in MR cholesterol composition may affect differentially $CD4^+$ and $CD8^+$ T-cells, the former being much more affected.

To further support the hypothesis that the properties of the signalosome in $CD8^+$ T-cells differ from that of $CD4^+$ T-cells we assessed the effect of MR disruption on T-cell proliferation. Data revealed that $CD8^+$ T-cells were less sensitive to a low concentration (0.5 mM) of β-methyl cyclodextrin, a MR disrupting agent, as compared to $CD4^+$ T-cells. Whereas the proliferative response of $CD4^+$ T-cells of young and elderly donors was completely abolished, there still remained a partial response of $CD8^+$ T-cells to TCR/CD28 stimulation. These observations suggested differential intrinsic properties of MR in $CD4^+$ T-cells as compared to $CD8^+$ T-cells which may result in a differential mode of signaling. These data may also explain the differential kinetic of IL-2 production by $CD4^+$ and $CD8^+$ T-cells.

One key component of MR is cholesterol which serves to stabilize their structure and to modulate their fluidity [19]. The concentration of cholesterol was 1.6-fold higher in MR fractions from $CD4^+$ and $CD8^+$ T-cells of elderly subjects as compared to young individuals. The anisotropy of $CD4^+$ and $CD8^+$ T-cells and MR fractions prepared from these cells was increased by approximately 10% in the case of elderly donors, suggesting an inverse correlation between MR cholesterol content and plasma membrane fluidity. The cause of the increase in the concentration of cholesterol in resting T-cells with aging is not known but may be the result of an imbalance in cellular cholesterol metabolism. Preliminary data from our laboratories indicate that significant changes occur with aging in the HDL-mediated reverse cholesterol transport. This mechanism is membrane raft-dependent [20] and suggests that its deregulation may contribute to the elevated plasma membrane cholesterol content in T-lymphocytes from normolipemic elderly humans. Altogether the increased cholesterol content and decreased fluidity of the membrane found here in both T-cells subsets reinforce our previous data in T-cells [21], contributing to functional decrease, however can not give an explanation for the differential functional behavior between these T-cells subsets. In this context another question arises concerning the properties of the lipids ordering the lipid rafts. We showed a quantitative increase in rafts cholesterol with aging but changes in oxidative status should alter MR properties and functioning as well. Since $CD8^+$ T-cells possess a cytotoxic activity via their granules, they may be gifted with a higher potency towards oxidation and other aggressions than $CD4^+$ T-cells also explaining why they were less affected by immune senescence. Moreover, unsatured fatty acids were shown to inhibit T-cells activation and functions by selectively displacing signaling molecules from MR. Thus, changes in fatty acids composition may also explain discrepancies between young and elderly donors as well as between $CD4^+$ and $CD8^+$ T-cells from the same donor. We are currently addressing these questions.

In view of the alterations in plasma membrane cholesterol concentration in MR, we also analyzed the distribution of the GM1. The GM1 fluorescence intensity in $CD4^+$ and $CD8^+$ T-cells of elderly individuals was more than two-fold than that measured in the corresponding T-cells of young donors. The increase in GM1 may have critical effects on T-cell functions that depend on MR, namely the recruitment of proteins involved in the early events of signaling. In this connection, it has been reported that over-expression of membrane microdomains constituent such as GM1 in PC12 cells can suppress nerve growth factor signals by modulating signal-transducing molecules localization and plasma membrane fluidity [22]. As a corollary, high levels of GM1 in MR of resting $CD4^+$ T-cells of elderly individuals may interfere with GM1 turnover [23] resulting in defects in early T-cell signaling as well as in IL-2 production.

The end-point of MR function is to induce the formation of the IS via SMAC [24, 25]. The data of O'Keefe et al. [26] showed that the formation of SMAC is not required for activation of naïve $CD8^+$ T-cells, giving support to the differential sensitivity of activation between $CD4^+$ and $CD8^+$ T-lymphocytes. This reinforces our hypothesis that $CD4^+$ T-cells did not behave in the same manner as $CD8^+$ T-cells in aging due do their differential mode of signalling. The triggering of CD28 is a critical step for MR polarization which results in SMAC formation leading ultimately to IL-2 production [27]. Differential alterations in the CD28 signaling between $CD4^+$ and $CD8^+$ T-cells

subsets may clearly explain the functional alterations of MR with aging leading to altered signaling and function mainly in $CD4^+$ T-cells, as will be described below.

2.4 T-cell Receptor Signalling and its Changes with Aging

T-lymphocyte activation culminates in cell proliferation and differentiation into effector and memory cells. The engagement of the receptors by duly presented antigens leads to a specific response driven by the signaling cascade. At the very early step of T-cell activation there are several key events that determine the specificity and the intensity of T-cell response. The first step in TCR-mediated signaling is the activation of different tyrosine kinases, leading to the tyrosine phosphorylation of several downstream molecules. The first signal through the TCR induces the phosphorylation of Lck, via recruitment of ZAP-70 leading to LAT phosphorylation (see Figure 1) which becomes a scaffold for the recruitment of multiple partners including other adaptor proteins and enzymes involved in phospholipid metabolism such as phosphatidylinositol-3-kinase (PI3K) and phospholipase-Cγ1 (PLC-γ1). A host of experimental data support the view that many proteins involved in T-cell signaling such as $p56^{Lck}$, LAT, SLP-76, protein kinase-Cθ and Gads are recruited in MR, whereas others such as CD45 are excluded [28] or transiently associated as in the case of CD4 [29].

The activation of Lck is a very tightly controlled process, which involves phosphatases, such as CD45 and the tyrosine kinases Csk, as well as regulatory molecules, such as Cbp/PAG and FynT. The control of Lck activation involves the tyrosine phosphatase CD45 and the PTK Csk which is regulated by the MR-resident Cbp/PAG and FynT, as well as the CaMKII substrate, cytosolic resident C3BP [30–32]. Csk is a ubiquitously expressed cytosolic PTK; it plays a negative regulatory role in cells by inhibiting intracellular processes induced by Src tyrosine kinases. The Csk SH2 domain interacts specifically with several tyrosine phosphorylated molecules and among them with the recently identified adaptor-Csk-binding protein/phosphoprotein associated with glycosphyngolipid-enriched microdomains (Cbp/PAG). Cbp/PAG has been shown to be palmitoylated and targeted to rafts. In resting human T-cells Cbp/PAG is constitutively phosphorylated and this results in recruitment of Csk to the rafts. This interaction increases the catalytic activity of Csk on its substrate, thereby inhibiting Src tyrosine kinases activity. However, this interaction is reversible. The dephosphorylation of Cbp/PAG releases Csk and promotes the activation of Src kinases upon TCR stimulation. This represents a sort of threshold regulator in T-cell activation. So far, no data exist concerning the activity of these factors with aging. However, it can be hypothesized that the interaction between Cbp/PAG and Csk is altered, therefore affecting the release of Csk.

With aging there is a well-known deregulation of the immune response. This deregulation is mainly the reflection of alterations in the cellular immune response mediated by T-lymphocytes. The main alterations are the decreased proliferation due to reduced IL-2 production leading to altered clonal expansion. The causes of this decline are not well understood, however, many explanations have been proposed.

One hypothesis to explain this observation suggests alterations in TCR-dependent signaling. During the past few years our laboratory has greatly contributed to the elucidation of the multiple changes in TCR signal transduction [16]. We and others have shown that several steps of the signaling cascade following TCR ligation are altered with aging [33]. However, much effort has focused on downstream events of T-cell signaling and less attention has been given to possible alterations in upstream events [34, 35], including the assembly of signaling molecules in MR. Recently, we have presented evidence that the age-related alterations in T-cell activation are linked to changes in MR composition and function [16]. It is now well documented that other early events related to protein tyrosine phosphorylation following TCR activation are altered in T-cells with aging, such as the generation of myoinositol 1, 4, 5-trisphosphate, intracellular free calcium mobilization, and PKC translocation to the membrane. It was also shown that defects in translocation of PKC following TCR stimulation are present in T-cells of old humans [15] and mice. Recently, our work showed that the activation, that is, tyrosine phosphorylation of the upstream molecules, such as Lck and LAT was also altered with aging. Thus, with aging we observe an alteration in all activation phases of T-cell signaling. This activation via the intermediate signaling events finally should lead to the activation of NFAT and NF-kB for the production of IL-2, which is consequently also altered with aging (Fig.1).

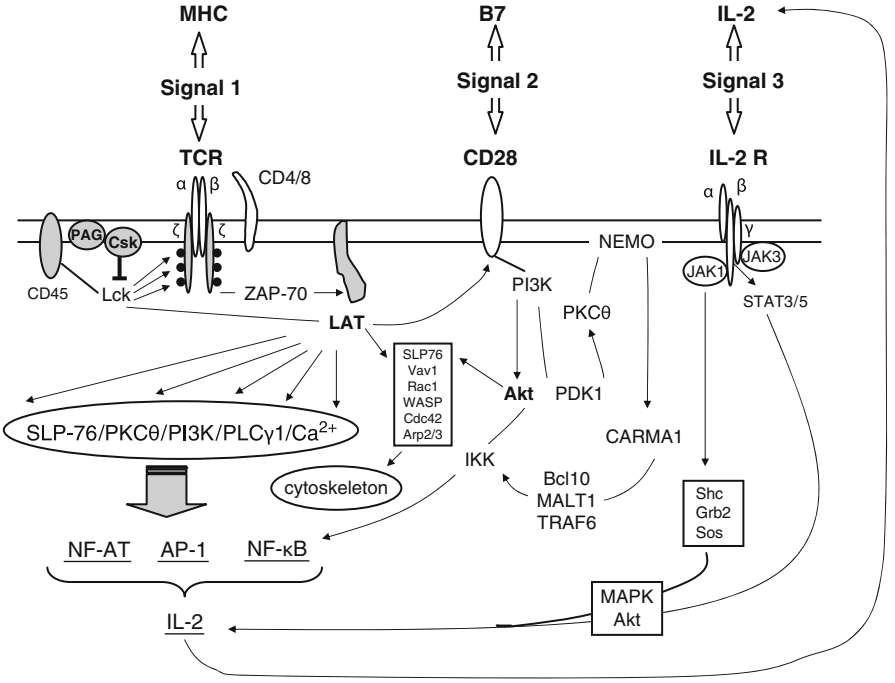

Fig. 1 Signalling pathways involved in signal 1, 2, and 3. TCR, CD28 and IL-2 receptor signalling is shown. The signalling events shown here are described and quoted throughout this review

Recently, data clearly showed that changes in MR machinery also occur in autoimmune diseases such as in systemic lupus erythematosus (SLE). Jury et al. [36, 37] demonstrated in their paper that $p56^{Lck}$ was over-associated in MR of $CD3^+$ T-cells explaining the hyperactivity of these cells in SLE patients. Based on the data presented, we suggest that the changes in MR composition and functions lead to impaired $p56^{Lck}$ activation and may be the main cause of the alterations in $CD4^+$ T-cell functions and consequently in immune senescence.

2.5 CD28 Dependent Signaling and Their Changes with Aging

For an efficient T-lymphocyte activation, the T-cell co-receptors (e.g., CD28, ICOS) should also be activated by their ligands (e.g., CD80/CD86) expressed on antigen presenting cells (APC) [38–41]. Certain pathways seem to be privileged and among them the phophatidylinositol 3-kinase (PI-3K; *See* Fig. 1). The main components of the PI-3K pathway include the following molecules PI-3K→Akt→IKK→NF-κB and PI-3K→PDK-1→PKCθ→IKK→NF-κB from which certain are recruited to MR. Interestingly, the CD28 pathway also activates the Lck, LAT, SLP-76, Grb2/GADS, Vav and the protein phosphatase PP2A [42–45]. Ultimately this co-stimulatory pathway regulate the translocation of NF-AT and NF-κB [46, 47]. The cytoplasmic tail of CD28 is phosphorylated by Lck which in turn initiate the recruitment and activation of PI-3K [48]. PI-3K initiates the translocation of Akt (PKB) in MR following its phosphorylation by PDK1. PDK1 is inserted in MR and phosphorylates PKCθ which leads to the activation of the latter molecule. The activated PKCθ recruit NEMO to MR and activates, via CARMA1 (CARD11), the complex Bcl10/MALT1/TRAF6 [43–45]. This complex induces the ubiquitination of IKK, its degradation by the proteasome and, finally the activation of NF-kB and the translocation of the Rel proteins to the nucleus. Thus, PI-3K and Akt are the essential early components for the induction of T-lymphocytes functions by the concurrent and/or individual activation of TCR et CD28 [45, 49]. All these events assure that IL-2 will be produced and secreted. As the level of CD28 expression is decreasing with aging this could contribute to the diminished production of IL-2 via an altered T-lymphocyte signaling, leading ultimately to a decreased T-lymphocyte clonal expansion.

Our recent work indicates that CD28 signaling leading to the phosphorylation of Akt is decreased mainly in $CD4^+$ T-cells from aged individuals. Akt was weakly activated in $CD4^+$ T-cells of elderly subjects but not in the case of $CD8^+$ T-cells. These data indicate a critical alteration in CD28 signaling in $CD4^+$ T-cells of elderly subjects, which can not be explained by the slight change in CD28 expression. Paradoxically, the marked increase in $CD28^{low}$ $CD8^+$ T-cells did not affect Akt activation. This further contributes to the decrease of NF-κB activation in mice and in humans already shown to be due to a decreased inactivation of IκB by the proteasome. Moreover, in view of the pleiotropic effects of Akt, its decreased activation also suggested that in $CD4^+$ T-cells, downstream signaling events including the up-regulation of the transcription factors, NF-kB and NF-AT, would be impaired and that would result in defects in

cytoskeletal rearrangements, cell cycling and ultimately in a decreased production of IL-2 in T-cells. We have also demonstrated that the CD28 number only partly explains the inability to activate adequately T-cells as Akt activation was more efficient in $CD8^+$ T-cells, having reduced CD28 co-receptors, compared to $CD4^+$ T-cells, having relatively conserved CD28 co-receptor number with aging. Our most recent results seemed to suggest that this is not the decreased number of CD28 co-receptors which plays the crucial role but the altered CD28 localization as a determinant factor of the immunosenescence [10]. Thus, changes in the CD28 co-receptor signaling might have far reaching consequences on T-cell functions in aging.

These findings explain one very important finding in immunosenescence which is the differential sensitivity of $CD4^+$ and $CD8^+$ T-cells towards activation induced cell death (AICD). $CD4^+$ T-cells are more susceptible to AICD than $CD8^+$ T-cells [50]. This can be explained by the differential signaling of CD28 towards Akt activation as this pathway mediates the survival/apoptosis of T-cells. Moreover, we already published data showing that the level of expression of a special receptor is not the best marker for cellular function but its differential membrane localization, such as for Toll-like receptors [51] and/or signaling molecules will ultimately influence cell fate and this immune function.

2.6 Convergence of TCR/CD28 Signaling Pathways in T-cells Activation and Their Changes with Aging

The signaling pathways elicited by TCR and CD28 converge for inducing the translocation of NF-κB and initiate the transcription of the IL-2 gene. It was suggested that the amplification of the signaling cascade initiated by the TCR is mainly dependent on CD28 for the polarization of MR [47]. Indeed, the engagement of CD28 induces the redistribution of MR enriched in GM1 at the site of TCR contact with APC. CD28 generates a favorable environment where the signals are protected, segregated and amplified. This prolonged physical stability between the T-lymphocyte and APC is fundamental to the production of IL-2 and to the clonal expansion of T-lymphocytes [40]. The IS formation is occurring after the MR polarization. The IS is a special spatial region highly organized containing signaling proteins, adhesion and cytoskeleton molecules [3, 24, 52, 53]. In this context, it is of note that the activation of $CD4^+$ and $CD8^+$ lymphocytes differ in their dynamics. The $CD4^+$ necessitate a prolonged activation to be able to proliferate, while a hour contact is enough in case of $CD8^+$ lymphocytes [54]. Three different studies including our suggest a differential role for MR in the activation of $CD4^+$ and $CD8^+$ cells [6, 10, 17] as described above.

In summary, with aging, there is an alteration of T-cell signaling either in signal 1 or signal 2 or both. As already described there are many alterations in the signaling cascade of T-cells, including calcium metabolism, tyrosine kinases phosphorylation, and PKC translocation to the membrane. Moreover, it is now well accepted that there are alterations in the very early stages of the signaling cascade, that is, in the composition and function of MR. There is an increase in cholesterol and sphingolipid content,

while a decrease in Lck and LAT tyrosine phosphorylation was observed. Not only was the composition of membrane raft found altered but also their functions. With aging MR polarize much less than do those of young subjects. These changes, taking into account what was described above concerning the role of MR in IS formation, underline the functional changes observed in T-cells with aging. It is of note that various subpopulations of T-cells are differently affected. $CD4^+$ cells are most affected by these signal transduction changes with aging, whereas although $CD8^+$ T-cells are also affected, their reactivity is better maintained than that of $CD4^+$ T-cells. Nevertheless, one should also consider the changes within CD8 and CD4 susbets, i.e., naïve versus memory cells. This will need further investigations to identify whether the loss of cellular functions and signaling are only due to loss of CD28 expression (in the memory cells) or has another origin.

2.7 IL-2 Signalling and its Changes with Aging

IL-2 is one of the most important cytokine for T-cells representing the "signal 3" for the efficient clonal expansion of T-cells under antigenic stimulation [55]. IL-2 receptor is composed of several subunits having specific role, however only the β subunit is involved in the signaling initiation [56, 57]. Whether this subunit is associated with MR for effective signaling is still controversial. Nevertheless, the signaling cascade is well known. The ligand attachment to the IL-2 receptor is initiating the activation by tyrosine phosphorylation of Janus kinases 3 (JAK3), which in turn activates the signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5) [58, 59]. This results in the translocation to the nucleus of these transcription factors which initiates the cellular response of proliferation [60]. The Jak/STAT pathway is a rapid intracellular communication system used by many cytokines and growth factors to mediate signals from the plasma membrane to the nucleus in order to regulate proliferation and differentiation of most tissue types (*See* Fig. 1). These pathways play a crucial role in the induction of the T-cell response to these cytokines namely clonal expansion [60, 61]. Many factors are controlling the Jak/STAT pathways which are also zinc dependent [62]. Furthermore, the Jak/STAT is one of the signaling pathway which is sensitive to redox conditions [63, 64].

In T-cells from elderly individuals we reported recently an alteration in IL-2 receptor signal transduction resulting in decreased JAK3 and STAT3/5 activation [65]. Thus, aging is accompanied with a signaling defect of the cytokine receptors IL-2 independently of the receptor number, as was already demonstrated [21] except for the very elderly aged over 90 years. This latter phenomenon seems to be in accordance with studies demonstrating less immune dysfunction in old old compared to young old individuals suggesting a contribution of an intact immune system to longevity. It is of note that zinc supplemented at physiological doses could not modulate the altered IL-2 signaling of individuals aged up to 90 years old. This suggests that either the normal zinc levels in T-cells are not sensitive to a supplementation or that the zinc mediated processes including anti-oxidant, anti-inflammatory, membrane physiology

maintenance are not major players in the altered signaling. Indeed, as we have shown, one of the basic age-related alterations affects the membrane composition [10]. In contrast, over 90 years old, the zinc could reverse the negative signaling effect of IL-2 indicating that the physiological behavior of T-cells of old–old individuals is fundamentally different. This needs further studies to determine the mechanism by which zinc is acting but it can be hypothesized that the inhibitory molecules like Protein inhibitor of activated STATs (PIAS) can be more efficiently modulated at this age [66]. This indicates that in T-lymphocyte activation one should always take into account the negative regulatory factors too.

3 Membrane Lipid Rafts and Cytoskeleton

T-cell activation involves F-actin rearrangements. Several molecules which are associated with DRM participate in tethering DRM to the actin cytoskeleton. Actin polymerization is regulated by the RhoGTPase Rac1 which activates WASP and Cdc42 which upreglulates the activity of Scar/WAVE. Activation of WASP and WAVE stimulates F-actin branching by upregulating the activity of the Arp2/3 complex [67]. The interaction between DRM and the actin cytoskeleton works in two directions: DRM-associated proteins regulate F-actin rearrangements whereas the actin cytoskeleton serves to induce and sustain DRM polarization in activated cells. During the formation of IS, CD28 is responsible for actin rearrangement and the coalescence of DRM. In addition, the adaptors Vav1 and Slp76 are key regulators of actin rearrangements required for the accumulation of signaling molecules/DRM at the T-cell/APC interface. The upregulation of Vav1 activity by CD28 is achieved through Lck. Thus, Lck is involved in CD28-related actin remodeling, MR coalescence and T-cell activation. A decade ago, it was found that F-actin polymerization was altered in T-cells of elderly under stimulation. No data in relation to MR exist, however, considering the alterations found in their composition and function in T-cells with aging we can suggest that the F-actin rearrangements could also be deficient in aging. Taken together, it can be concluded that with aging there is an alteration in T-cell activation due to a deregulation of the intracellular signaling pathways via an alteration of the T-cell membrane composition leading to altered functions, such as proliferation and IL-2 production. Although F-actin polymerization has been reported to be altered in lymphocytes of aged mice [68], there are no data in T-cells with respect to actin reorganization in young or elderly subjects and the relationship to MR.

4 Negative Regulation of T-Lymphocyte Activation and its Changes with Aging

Lymphocytes are not only positively activated by kinases but also negatively. This can be at the level of various molecules of the signaling cascade or the termination of the activation process. One way to negatively regulate T-lymphocyte activation is

through protein phosphatases (PPases). In addition to the kinase component of T-cell activation, it exists other enzymes which intervene in the negative regulation of the signaling cascade such as the PPases and the phosphatidylinositol (PtdIns) phosphatases [69, 70]. The most important targets of PPAses are the activation pathways of Lck and PI-3K. SHP-1 dephosphorylates and inhibits PI-3K [47, 71]. The PPase SHIP and the PtdIns phosphatase PTEN converge for the negative regulation of PI-3K. While SHIP hydrolyses the phosphate groups on phosphotyrosine residues of PI-3K, the PTEN cut the phosphate groups in position 3 of PtdIns-3, 4, 5 trisphosphates, destroying recognition site by the PH domain of PI-3K [71–73]. The activity of PPases, which is as finely regulated as that of protein kinases, ultimately also depends on their interaction with MR. This is clearly demonstrated for the modulation of CD45 activity [74–76] and as we have demonstrated for the PPase SHP-1 [77]. CD45, when located in MR has a positive effect on Lck activation, while when CD45 is displaced, such as in the quiescent state, Lck is inactivated. We have recently shown a similar phenomenon for SHP-1 in neutrophils [77]. There is more and more experimental evidence that the balance between tyrosine kinases and phosphatases is essential for the maintenance of the resting status and for activation, which can predict alterations with aging. Only a few data exist concerning phosphatase activity in T-cells with aging. CD45 is a receptor-like phosphatase expressed on all nucleated hematopoietic cells. One key function of CD45 is to serve as a positive regulator of Src tyrosine kinases, by opposing Csk function, and dephosphorylating the negative regulatory C terminal tyrosine of Src tyrosine kinases. CD45-protein tyrosine phosphatase activity in old T-cells was found to be decreased compared to young cells [78]. However, it may be necessary to reassess the behavior of CD45 under activation in terms of its involvement in the IS, from which it is usually excluded upon T-cell activation. Our own studies using cholesterol repletion of T-cells from young subjects, being a partial aging model of T-cells, suggest alterations in phosphatase activities (our unpublished data). Furthermore, because in neutrophils which are very short-lived cells important alterations were found for SHP-1 activity, it can be suggested that phosphatase activities might also be altered with aging in long-lived cells such as T-cells. Our very recent data indicate that there is much more SHP-1 content in the membrane of T-cells from elderly compared to young subjects [79]. The activity of SHP-1 is also increased in T-cells of elderly as determined by tyrosine phosphorylation following anti-CD3 and anti-CD28 stimulations compared to identical conditions in T-cells of young subjects. The exact significance of this increased activity is not well understood, but could have a negative effect on Lck activation [80]. Altogether, there are interestingly very few data concerning the phosphatase activity in relation to TCR activation. Certainly, no data exist concerning their association/recruitment to MR. This should be further explored in the future.

The other way to negatively regulate T-lymphocyte activation is by scaffold Homer proteins. The Homer proteins are composed of three members. These proteins expressed in several tissues were found in MR of glial cells. These Homer proteins were, until very recently, associated to Ca^{2+} mobilization, following their interaction with TRP canonics (TRPC) [81]. However a recent publication of Huang and al [82] clearly demonstrate that Homer2 and Homer3 are negative regulators of lymphocyte activation. These proteins compete with calcineurin for NF-AT. This

competition stops the calcineurin-dependent dephosphorylation of NF-AT and its subsequent translocation to the nucleus. These results raise the possibility that Homer (1, 2 ou 3) could be differentially recruited in MR of lymphocytes in elderly subjects. A preferential and sustained recruitment of Homer in MR could contribute to the diminution of the immune response of elderly subjects.

Thus, most of the early signaling events were shown to be altered with aging in human T-cells especially in $CD4^+$ T-cells. Thus, it would be very difficult to assign the alteration in T-cell activation to any of the participating signaling molecules. Then, what can be the cause of these signaling alterations in T-cells occurring during immunosenescence? Could a common change explain this signaling alteration in T-cells upon activation? Investigations in the late 1980s already suggested that biochemical and biophysical alterations of the cell membrane could be responsible for the altered immune response with aging. Alterations in the lipid composition and fluidity of the cell membrane were found [83]. One explanation that is naturally emerging is the changes at the membrane level either qualitatively or quantitatively.

5 Membrane Composition Changes with Aging: Role of Cholesterol

It was suggested several decades ago that the T-cell membrane from elderly subjects is more rigid than that of young subjects [83]. We recently presented evidence that an increase in free cholesterol could explain these physicochemical changes observed about 20 years ago [19]. There is a twofold increase in the T-cell membrane cholesterol content with aging. Cholesterol is an essential component of the membrane as it maintains a certain order in the plasma membrane structure, as it is now well recognized, through the MR (*See* Fig. 2). This increase in cholesterol content leads to the contention that if we can extract the overcharge we would be able to restore T-cell functions. Unfortunately, until now only partial restoration of the functions was obtained. Methyl-β-cyclodextrin (MBCD) used in small quantities has so many other membrane disturbing effects that no functional improvement was observed in T-cells of elderly [21, 84]. Statin (which inhibits cholesterol synthesis via the inhibition of the HMG-CoA reductase) used at high concentrations necessary to see a reduction in cellular cholesterol levels in Jurkat cells resulted in apoptotic death [51]. The only known physiological cholesterol extracting agent is high-density lipoprotein (HDL). HDL via the reverse transport of cholesterol is able to decrease the membrane cholesterol content very rapidly, but it was much less efficient in case of T-cells of elderly (our unpublished data). Nevertheless, the proliferation and IL-2 production of T-cells of elderly were slightly improved.

The other way to assess the role of cholesterol is to replenish the membrane of T-cells of young subjects with cholesterol to the level observed in T-cells of elderly subjects. Our results with the replenishment with free cholesterol have shown a decrease in proliferation and IL-2 secretion, such as observed in immunosenescence. Thus, increased cholesterol in the membrane of T-cells from young subjects

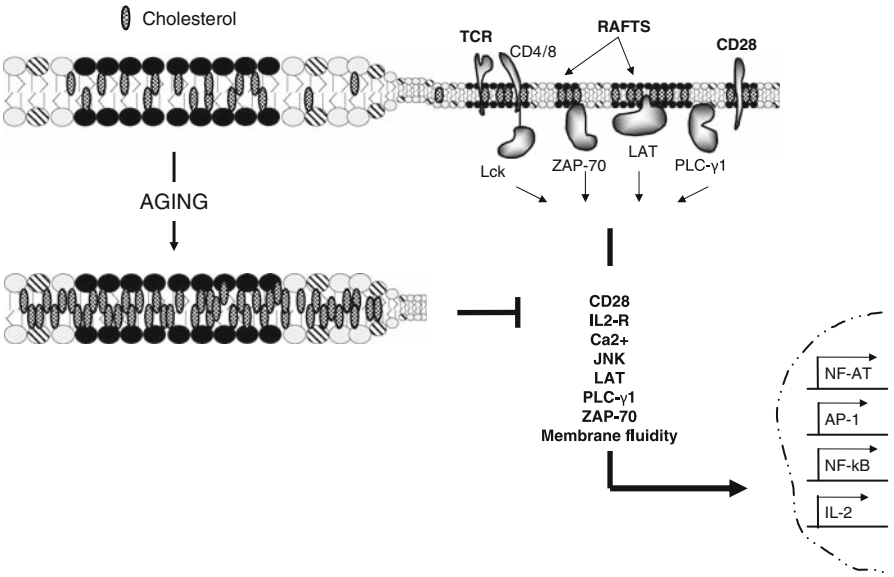

Fig. 2 Age-associated alterations in TCR signaling. TCR signaling events which are altered with aging are depicted here. Non-cited molecules or pathways are not changing with age. The relevant reference can be find throughout this review

rendered them functionally aged. In the mean time the GM-1 content in the membrane is increased. There is no explanation why the cholesterol is increasing as the serum cholesterol content is remaining unchanged in elderly subjects. It could be that the cholesterol uptake is dysregulated, the intracellular cholesterol production via the HMG-CoA reductase can be increased, or that the reverse cholesterol transport assured by HDL is deficient. Our recent experimental data seem to indicate that the reverse transport of cholesterol by HDL is indeed altered in T-cells with aging.

6 Do Membrane Rafts Properties Contribute to Human Immunosenescence?

Considering all the changes described above the question naturally arises what is the role of MR and could changes in their composition and in their function contribute to the altered T-cell activation observed during immunosenescence? The experimental data presented so far seem to support a positive answer to this question. With aging, as described above, we demonstrated an alteration in the function of the MR as they are almost unable to coalesce in $CD4^+$ T-cells with aging. The alterations are less dramatic for $CD8^+$ T-cells. We have demonstrated an alteration in the recruitment and activation of Lck and LAT into MR. In this context one of the most important findings is that the CD28 as well as the IL-2R cannot be recruited to the membrane rafts in $CD4^+$ T-cells of elderly subjects explaining

the alteration of the signaling of these receptors with aging. In contrast, in $CD8^+$ T-cells these receptors are already recruited to the MR. Thus, the age-associated alterations in their properties include the increase in cholesterol content, impaired coalescence, and selective differences in the recruitment of key proteins involved in TCR signaling. It can be thus hypothesized taking into account these experimental data that the increased rigidity of the membrane following the increase in cholesterol content limits MR functionality. This loss of function leads to the inability to recruit to the IS the necessary machinery or alternatively to exclude the nonparticipating molecules to reach an adequate activation, which is a hallmark of immunosenescence.

7 Conclusion

With aging we observe an alteration of the immune response collectively designated as immunosenescence. One of its most striking aspects is the altered T-cell activation for clonal expansion by specific antigens. The causes of this decreased activation are not completely known. Recent studies shed light on the role of signaling alterations following TCR and CD28 ligation. The final outcome of protein rafting is the formation of the IS, which is needed to sustain the activation, which will result in a proper immune response. We can document changes in molecular events with aging, but we are not yet able to explain these changes. The ultimate defect in signaling can be explained by the newly discovered membrane rafts alterations in composition, function, and size with aging. These functional and physicochemical properties are influenced by intrinsic as well as extrinsic factors. Understanding the events that lead to changes in the TCR signaling cascade would be of great benefit considering the large number of diseases in which MR dysfunction is thought to play a role. Altogether these data suggest that MR alterations in T-cells do contribute to immunosenescence.

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Molecular Signaling of CD95- and TNFR-Mediated Apoptosis in Naïve and Various Memory Subsets of T-Cells

Sudhir Gupta and Ankmalika Gupta

Contents

Abstract: There are multiple ways for cells to die, including necrosis, apoptosis, and autophagy. Apoptosis or programmed cell death or suicidal cell death is a physiological form of cell death, which is critical in cellular homeostasis. Apoptosis occurs in almost all cell types in the body and begins as early as eight cell embryo stage and continues throughout the lifespan of the organism, albeit at different rate. There are multiple roads to apoptotic cell death, including extrinsic or death receptor-mediated and intrinsic, which may be mediated via mitochondrial pathway and the endoplasmic reticulum pathways. Most of apoptotic cell death are mediated by serine proteases, the caspases, which cleave a number of target substrates, including enzymes, transcription factors, and structural proteins. However, apoptosis may also be mediated by caspase-independent pathways. In this review we will discuss molecular signaling and regulation of death receptor pathways, particularly CD95- and TNFR- mediated apoptosis, in naïve and various memory subsets of T-cells, and changes during human aging.

Keywords: CD95 • Caspases • NF-κB • FLIP • TNF • TNF receptors

S. Gupta (✉) · A. Gupta
Medical Sciences I, C-240
University of California, Irvine
Irvine, CA 92697
Tel.: (949) 824-5818
Fax: (949) 824-4362
E-mail: sgupta@uci.edu

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1 Introduction

Life requires death; without cell death many of us may have been borne with chromosomal defects. There is an evidence to suggest at even at 8 cell stage of embryo, 2 cells that display chromosomal abnormalities are deleted by apoptosis. In postnatal life, apoptosis plays a critical role in cellular homeostasis and removal of mutated or undesired cells. In the immune system, apoptosis plays an important role in selection of T-cell repertoire, killing of target cells by cytotoxic T-cells (CTL) and natural killer cells, removal of effector cells at the termination of an immune response, immune privilege, and lymphocyte homeostasis. One of the major players in the execution of apoptosis is a group of cysteine proteases, the caspases; though under certain conditions, and in certain cell types, apoptosis may be mediated by a caspase-independent pathway (Loeffler et al. 2001). Apoptosis signals may be mediated via extrinsic or death receptor pathway (Ashkanazi and Dixit 1998; Gupta 2001, 2002; Larvik and Krammer 2005; Gupta and Gupta 2007), and intrinsic pathway, which is mediated via mitochondria and the endoplasmic reticulum (ER) (Ferri and Kroemer 2001; Gupta 2000; Gupta and Gupta 2007; Green and Evan 2002; Kroemer and Reed 2000; Martnou and Green 2001; Zamzami and Kroemer 2001). In all 3 pathways, a set of distinct initiator or proximal caspases are activated, which then activate common effector or executioner caspases to induce morphological and biochemical features of apoptosis (Gupta 2002). All caspases are produced as catalytically inactive zymogens and undergo proteolytic activation. Initiator caspases (caspase-8 and caspase-10) are activated in a large membrane death-inducing signaling complex (DISC). Initiator caspases are characterized by the presence of 80–100 amino acid death domain (DD). DD superfamily is comprised of subfamily of DD, death effector domain (DED), and the caspase-recruiting domain (CARD), which facilitates the recruitment of initiator caspases into the DISC. Initiator caspases undergo autoproteolytic activation following homodimerization. Activated initiator caspases cleave and activate executioner caspases, primarily caspase-3, caspase-6, and caspase-7. Activated executioner caspases cleave a number of cell-death substrates, including actin, lamin, inhibitor of caspase-activated DNAse (ICAD), plectin, RAS homologue-associated coiled-coil containing protein kinase 1 (ROCK1) and gelsolin, DNA-repair enzymes, and survival transcription factors to induce apoptosis (Gupta 2002; Igney and Krammer 2002). The apoptotic cells express several “eat-me” signals including phosphatidyl serine and different surface sugars which allow them to be engulfed by neighboring phagocytic cells. More recently, certain caspases have shown to be involved the activation and proliferation of T-cells. However, these mechanisms will not be discussed in this review.

2 Death Receptor Signaling Pathways of Apoptosis

In the death receptor pathway, apoptosis cascade is triggered by signals via cell surface death receptors, which belong to a large superfamily of tumor necrosis factor receptors (TNFR), and are characterized by the presence of DD in their cytoplasmic

tail. They include TNFR1, CD95, TNF-related apoptosis-inducing ligand receptor-1 (TRAILR1), TRAILR2, death receptor 3 (DR3) and DR6 (Krammer et al. 2007). In this review we will discuss two prototype death receptors, the CD95 and TNFR.

2.1 CD95-mediated Apoptosis Signaling (Fig. 1)

Ligation of CD95 with CD95 ligand (CD95L) or anti-CD95 antibodies triggers the recruitment of a set of adaptor molecules and procaspases (due to homotypic interactions between their DD and DED) resulting in the formation of DISC. DISC

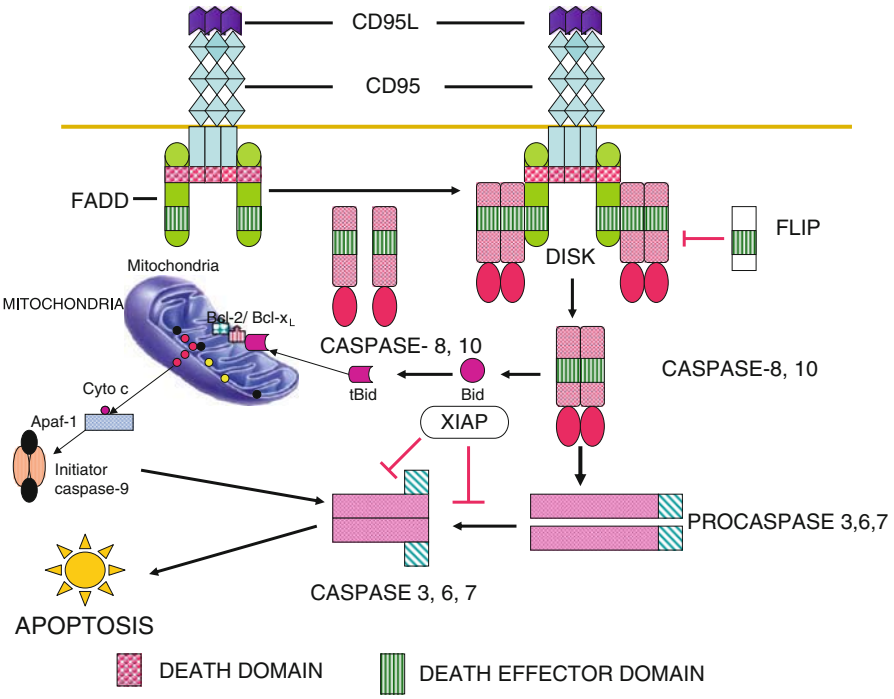

Fig. 1 CD95-mediated apoptosis pathway
Upon ligation with CD95 ligand (CD95L), CD95 undergoe oligomerization of its death doman (DD), which recruits am adaptor Fas-associated death domain (FADD) and then by homotypic protein-protein interaction between their death effector domain (DED), it recruits initiator pro-caspases (-8, -10) forming a death-inducing signal complex (DISC) as a platform for initiation of apoptosis, Procaspase-8, -10 are activated by homodimerization and axtive caspase-8, -10 are released from the DISC into the cytoplasm where they cleave executioner caspases to form homodimetic active executioner caspases to induce apoptosis. When caspase-8 at the DISC is low, it cleaves Bid to generate truncated Bid (tBid), which is translocated to the mitochondria where it promotes apoptosis by releasing cytocrome c. Cytochrome c binds to Apaf-1 and recruits procaspase-9 to form an Apoptosome. Active caspases-9 activates effector caspases resulting in apoptosis. XIAP inhibits the activation and activity of caspase-3.

contains oligomerized/trimerized CD95, Fas-associated death domain (FADD), 2 isoforms of procaspase-8, procasapse-8a (FLICE or MACHα1) and procaspase-8 (MACHα2), procaspase-10, and cellular FLICE inhibitory protein (FLIP). The formation of DISC results in autoproteolytic activation of initiator caspases, procaspase-8 and procaspase-10. The activation of procaspase-8 is dependent upon its local concentrations (high concentrations favor) for autoproteolytic activation. The homodimers of procaspase-8 have proteolytic activity and proteolytic process appears to occur at the DISC by 2 cleavage events, resulting the generation of an active caspase-8 tetramer (Chang et al. 2003), which is subsequently released from the DISC into the cytosol to activate effector procaspases to induce apoptosis. Procaspase-10 forms active heterodimer at the DISC; however, whether caspase-10 can trigger CD95-induced apoptosis in the absence of caspase-8 is controversial; levels of procaspase-10 at the DISC are not sufficient to trigger apoptosis alone (Kischkel et al. 2001; Sprick et al. 2002). Based upon the concentration of caspase-8 at the DISC CD95-mediated apoptosis signaling pathway is divided into 2 types (Scaffidi et al. 1998). Active caspase-8 concentration at the DISC is high in Type-I cells. In these cells, active caspase-8 activates effector caspase-3, caspase-6, and caspase-7. In contrast, Type-II cells are characterized by low levels of active caspase-8 at the DISC and requires additional amplifying mechanism to induce apoptosis. It involves cleavage of BH3-interacting-domain death agonist (BID) by active caspase-8 to generate truncated BID (tBID), which induces aggregation of Bcl-2-associated X protein (Bax) at the mitochondria and release of cytochrome c. In the cytosol cytochrome c binds to adapter Apaf-1 (Apoptosis-activating factor) to form large protein complex, apoptosome, along with procaspase-9. This is followed by activation of procaspase-9 to active caspase-9, which in turn activate effector caspase-3, caspase-6, and caspase-7 to induce apoptosis. Type-II signaling is blocked by Bcl-2 and Bcl-x_L, whereas Type-I signaling cannot be blocked by Bcl-2 or Bcl-x_L (Scaffidi et al. 1998). CD95-mediated apoptosis in T-cells is predominantly mediated via Type-I signaling.

2.2 TNFR-mediated Apoptosis Signaling

TNF-α exerts a variety of biological effects, including production of inflammatory cytokines, proliferation, differentiation, and cell death (Ashkanazi and Dixit 1998; Gupta 2000, 2001, 2002; Hsu et al. 1996). While pleiotropic effects of TNF-α are mediated by binding to type I and type II receptors (TNFR-I and TNFR-II), the death-inducing signal is predominantly mediated via TNFR-I; however, TNFR-II have been shown to participate indirectly in TNF-α-induced cell death via regulating apoptosis mediated by TNFR-I (Declercz et al. 1998; Haridas et al. 1998; Locksley et al. 2001; Pimentel-Muinos and Seed 1999; Screaton G and Xu 2000; Tartaglia et al. 1993; Thomas et al. 1990; Vandenabeele et al. 1995; Weiss et al. 1998). Both cell survival and cell death signals mediated by TNFR require distinct sets of adapters and other downstream signaling molecules. Steps of TNF-α -induced signaling are reviewed [33,34] and shown in Fig. 2.

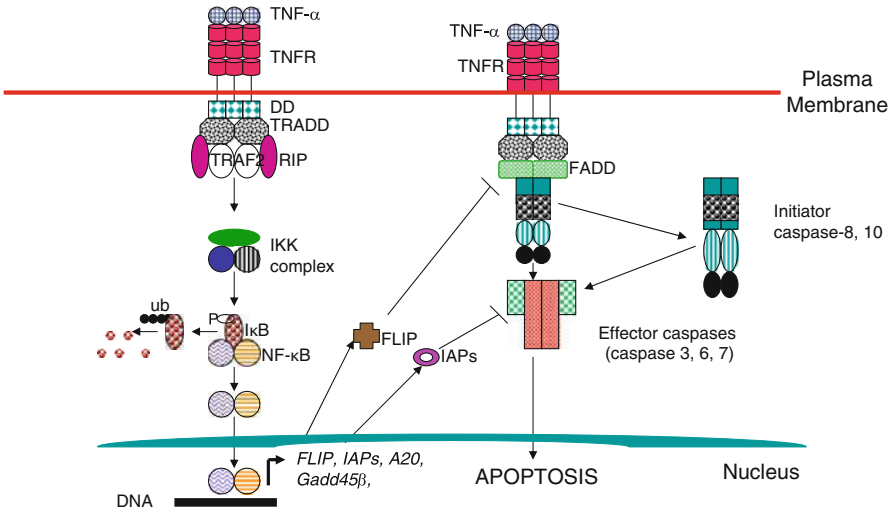

Fig. 2 TNF-TNFR signaling pathway
Upon ligation with TNF-α, TNFR-I mediates both survival signal and death signal by recruitment of different set of adapter proteins (TRAF-2/RIP and FADD).

Upon interaction with TNF-α, TNFR-I undergoes trimerization of its receptor death domains, which in turn recruit an adaptor protein, TNFR-associated death domain (TRADD). In order to induce death signal, TRADD recruits FADD. Therefore, for death inducing signaling via CD95 or TNFR, FADD serves as a common conduit. The remaining downstream signaling steps are similar to those described above for CD95-mediated apoptosis. Alternatively, for the survival and other biological function of TNF-α, TRADD recruits distinct sets of adapter proteins, the TRAF-2 (TNFR-associated factor-2) and receptor interactive protein (RIP). TRAF-2 and RIP stimulate pathways leading to activation of MAP kinase and NFκB. Both NF-κB (Ghosh and Karin 2002; Karin and Lin 2002) and transient activation of (mitogen-activated protein kinases) MAPK induce survival signals (Natoli et al. 1997), whereas prolonged activation of MAPK promote apoptosis (Hacki et al. 2000). MAPK is a family of proteins, including p38, MAPK, and extracellular signal regulatory kinase 1 and 2 (ERK). The antiapoptotic genes that are up-regulated by NF-κB activation include *cIAP1, cIAP2, XIAP, Gadd45*β, *Bcl-*X_L*, A20, TRAF-1, TRF-2 and FLIP* (Chen et al. 2000; DeSmaele et al. 2001; Ghosh and Karin 2002; Tang et al. 2001).

3 Regulation of Death Receptor-mediated Apoptosis

3.1 cFLIP Proteins

A role of cFLIP proteins in the inhibition of extrinsic pathway of apoptosis is well established (Golks et al. 2005; Thome and Tschopp 2001). Three alternatively spliced

forms of cFLIPs ($cFLIP_L$, cFLIPs, $cFLIP_R$) have been described. All 3 isoforms contain 2 DED with homology to n-terminal domain of procaspase-8 and are recruited to the CD95 DISC by protein-protein interactions via their DED. Both cFLIPs and $cFLIP_R$ are structurally related and block activation of procaspase-8 at the DISC; however, the role of $cFLIP_L$ is more complex. At high level $cFLIP_L$ blocks the activation of procaspase-8 at the DISC by blocking its processing, whereas at low concentration of $cFLIP_L$ at the DISC promotes the cleavage of procaspase-8 resulting in the formation of $cFLIP_L$—procaspase-8 heterodimer resulting in facilitating apoptosis. Therefore, $cFLIP_L$ based upon its concentration at the CD95 DISC may serve as antiapoptotic or proapoptotic molecule. Both cFLIPs and $cFLIP_R$ rescue T-cells from activation-induced cell death (AICD). A role of cFLIP in T-cell activation has been supported in FLIP transgenic and knock out mice, as well as by an overexpression of $cFLIP_L$ (Dohrman et al. 2005; Thome and Tschopp 2001).

In addition to 3 spliced isoforms, 2 N-terminal cleavage products of cFLIP, p43-FLIP and p22-FLIP have been reported, which promote survival via activation of NF-κB (Golks et al. 2005; Kataoka and Tschopp 2004). P43-FLIP (a cleaved form of $FLIP_{L)}$ activates NF-κB via its interaction with TRAF1, TRAF2 and RIP-1, which together activate NF-κB. Since FLIPs does not associate with this complex, it appears that caspase-like domain of cFLIPl is essential in the activation of NF-κB via TRF2/RIP pathway. P22-FLIP, which is generated by N-terminal cleavage by caspase-8, activates NF-κB by directly interacting with IKK complex via IKKγ. The ratio between procaspase-8 to cFLIP is critical in determining the amount of p22-FLIP generation and therefore, activation of NF-κB. Though described originally as an inhibitor of CD95-mediated apoptosis, it is apparent that cFLIP proteins also regulate TNFR-mediated apoptosis.

3.2 NF-κB

The predominant form of NF-κB in lymphocytes is a heterodimer comprising of p50 and p65. In unstimulated cells, NF-κB is kept in the cytoplasm through interaction with the inhibitory proteins termed as IκB (inhibitor κB) (Ghosh and Karin 2002; Karin and Lin 2002). When cells are exposed to TNF-α, IκB is phosphorylated followed by ubiquitination and degradation of IκB by the 26S proteosome. Free NF-κB dimers are released and translocated to the nucleus, where they activate transcription of a number of target genes, including anti-apoptotic genes *cIAP1, cIAP2, XIAP, Gadd45β, Bcl-X_L, A20, TRAF-1, TRF-2 and FLIP*.

3.3 A20 and Gadd45β

TNFR-mediated apoptosis is also regulated by A20 and gadd45β (De Smaele et al. 2001; Opipari et al. 1992). A20 is a ring finger protein, which has dual activity in

that it inhibits apoptosis as well as inhibits NF-κB activation (Heyninck and Beyaert 2005; Opipari et al. 1992).These activities of A20 are cell type specific. A20 inhibits NF-κB activation and therefore promotes apoptosis by first deubiquitination of K^{63} RIP (which activates NF-κB) and subsequent K^{48} ubiquitination of RIP rendering RIP susceptible to S26 proteasomal degradation. In contrast, A20 inhibits apoptosis, at least partially, by binding to TXBP151, which inhibits TNF-α-induced apoptosis. Furthermore, A20 and cIAP interact with a common region in TRAF2. Therefore, A20 releases cIAP from the TRAF2-signaling complex, thereby allowing cIAPs to exert their antiapoptotic effects. Gadd45β inhibits TNF-α-induced apoptosis by inhibiting prolonged activation of MAPK (De Smaele et al. 2001).

3.4 IAP Proteins

IAP family proteins, which were originally identified in the genome of baculovirus, have a key role in the negative regulation of caspase-dependent apoptosis mediated by death receptor, the ER pathway, and mitochondrial pathway (reviewed in Salvesen and Duckett 2004). The cIAP-1 and cIAP-2, two structurally homologous proteins were initially isolated by their interaction with TRAF-1 and TRAF-2 in the TNF-RII complex. cIAP1 is also recruited to DISK of TNF-RI by TRAF-2. In addition to cIAP1 and cIAP2, XIAP have a conserved COOH-terminal RING finger, zinc-binding domain (Liston et al. 1996). Among these IAPs XIAP suppresses apoptosis by preventing the activation of procaspases9-and caspase-3 and by inhibiting directly the enzyme activity of mature caspases. TRAF-2-IAP complex inhibits caspases-8 activation by an unknown mechanism.

4 Apoptosis in Naïve and Memory Subsets of CD4+ and CD8+ T-cells in Aging

4.1 T-cell Differentiation into Memory Subsets

Recent work has suggested that following virus infection or antigen stimulation, naïve T-cells (T_N) undergo a series of proliferative and differentiation steps ultimately culminating in an acquisition and maintenance of memory for a particular antigen/pathogen (Gupta et al. 2004; Kataoka et al. 2001; Sallusto et al. 2004; Tomiyama et al. 2002; Weninger et al. 2001). The memory T-cells display differential expression of adhesion molecules (CD62L) and chemokine receptors (CCR-7). CCR7+ and $CD62^{high}$ T-cells are found in lymph nodes, whereas CCR7- and $CD62L^{low}$ are found in extranodal sites such as liver and lung. Based upon these adhesion molecules and chemokine receptors, memory CD8+ T-cells have been divided into "central" memory (T_{CM}) T-cells for those that are found in lymphoid organs and

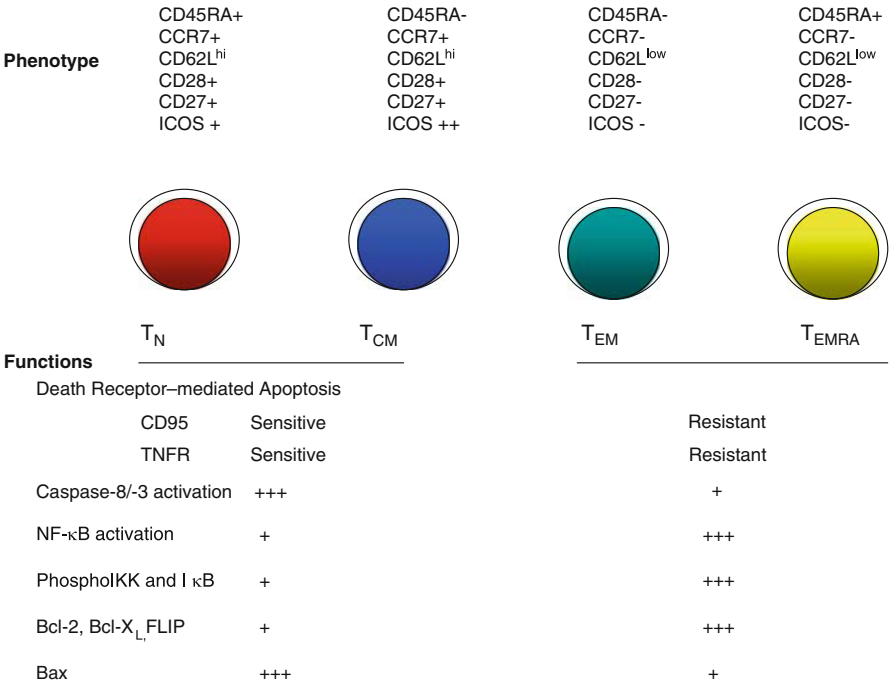

Fig. 3 Phenotypic and apoptosis characteristics of naïve (T_N) and different subsets of memory T-cells (T_{CM}= central memory; T_{EM} and T_{EMRA}= two types of effector memory)

"effector" memory (T_{EM}) cells that are found in peripheral nonlymphoid tissues and mucosal sites. Furthermore, effector memory CD8+ T-cells in humans (not in mice) have been further subdivided, based upon the expression or lack of CD45RA, into CD8+CD28-CCR7-$CD62^{low}$ CD45RA- (T_{EM}) and CD8+CD28-CCR7-$CD62^{low}$ CD45RA+ (T_{EMRA}). These subsets have been extensively characterized (Gupta et al. 2004, 2006) and shown in Fig. 3.

4.2 Apoptosis of Naïve and Memory Subsets and Effect of Age

We have reported that T_N and T_{CM} T cell subsets (T_{CM}>T_N) are sensitive to TNF-α-induced (Gupta and Gollapudi 2006, 2006a; Gupta and Gupta 2007), where as T_{EM} and T_{EMRA} cells are resistant to apoptosis. Similar observations have been made with CD95-mediated poptosis (Gupta et al. 2008), and oxidative stress-induced apoptosis (Gupta et al. 2007). We have also defined various molecular mechanisms responsible for such differential sensitivity to TNF-α-induced apoptosis (Gupta et al. 2006). Several investigators have reported increased apoptosis in T-cells and CD4+ and CD8+ T-cells in aged humans (Aggarwal and Gupta 1998;

Aggarwal et al. 1999; Gupta 2000; Gupta 2002; Gupta 2002a; Gupta et al. 2003; Lechner et al. 1996; Phelouzat et al. 1996, 1997). We and others have reported increased susceptibility of aged T-cells and CD4+ and CD8+ T-cells to activation-induced apoptosis and Fas (CD95)-induced apoptosis (Aggarwal and Gupta 1998; Aggarwal and Gupta 1999; Iwai et al. 1994; Lechner et al. 1996; Miyawaki et al. 1992; Phelouzat et al. 1997; Shinohara et al. 1995), which is associated with increased caspase activation (Aggarwal and Gupta 1999). Furthermore, we have demonstrated that FADD plays an important role in increased sensitivity of aged T-cells to apoptosis (Gupta et al. 2004). We have also demonstrated that TNF-α-mediated apoptosis is increased in both CD4+ and CD8+ T-cells in aged humans [Aggarwal et al. 1999; Gupta 2002; Gupta et al. 2003]. Increased apoptosis is associated with decreased expression of Bcl-2 and TRAF-2. We have demonstrated that both up-regulation of FADD and decreased NF-κB activity play an important role in increased sensitivity of aged T-cells to TNF-α-induced apoptosis. Recently, we have observed that T_N and T_{CM} CD8+ T-cells and CD4+ in aging are more sensitive to TNF-α induced [Aggarwal and Gupta 1998; Gupta 2002a; Gupta and Gollapudi 2006a; Kataoka and Tschopp 2004; Phelouzat et al. 1997], and anti-CD95-induced apoptosis (Gupta et al. 2008) as compared to young subjects. Therefore, increased sensitivity of T_N and T_{CM} CD8+ T-cells is not unique to TNF-α. We have also shown that T_{EM} and T_{EMRA} CD8+ and CD4+ T-cells in aged humans are equally resistant to apoptosis as that of young subjects, suggesting that the accumulation of T_{EM} and T_{EMRA} during aging is not due to alterations in apoptosis (Gupta and Gollapudi 2006, 2006a).

We and others have investigated various mechanisms, which may be associated with increased apoptosis of T-cell subsets in aged humans, especially TNF-α-induced apoptosis. NF-κB is an important regulator of TNF-α-induced apoptosis. Pahlvani and Harris (1996) reported decreased NF-κB DNA binding activity in nuclear extracts of concanavalin A-stimulated splenic lymphocytes from old Fischer rats as compared to young rats. Whisler et al. (1996) reported decreased levels of NF-κB in unstimulated and PHA, PMA and anti-CD3-stimulated T-cells from a small number of aged humans as compared to young subjects. Trebilcock and Ponnappan (1996) demonstrated decreased induction of NF-κB in response to PMA and TNF-α. These authors suggested that decreased induction of NF-κB could be due to decreased proteosome-mediated degradation of IκB (Ponnappan et al. 1999). We have investigated TNF-α signaling pathway of apoptosis in aged subsets in detail and observed that TNF-α-induced activation of NF-κB in T_N and T_{CM} is significantly decreased as compared to young subjects (manuscript in preparation). Furthermore, aged T_N and T_{CM} CD8+ T-cells display decreased activation of IKKα/β, and decreased phosphorylation of IκB as compared to T_N and T_{CM}. We have also demonstrated that an overexpression of IKKβ that resulted in the upregulation of NF-κB corrected increased sensitivity of aged T-cells to TNF-α-induced apoptosis (Gupta et al. 2005). Therefore, establishes a role of decreased NF-κB in increased sensitivity of aged T-cells to TNF-α-induced apoptosis.

5 Naïve, Central Memory and Effector Memory CD8+ T-cells in Aging

In aging, there is a significant reduction in naïve CD8+ T-cells (Fagnoni et al. 2002) and CD8+ CD28+ T-cells, which contain both naïve and central memory CD8+ T-cells (Brzeznska et al. 2004). In addition, there is an accumulation of CD8+CD28- T-cells, which are oligoclonal and show characteristics of cellular senescence (i.e., short telomere length indicative of long replicative history), and increased IFN-γ production [Bandres et al. 2000; Effros. 1994; Monteiro et al. 1996; Nociari et al. 1999; Posnett et al. 1994; Saurwein-Teissl et al. 2002]. These CD28- CD8+ T-cells are comprised of 2 subpopulations of effector memory CD8+ T-cells, namely T_{EM} and T_{EMRA} CD8+ T-cells. Our study shows a marked decrease in the proportions of naïve CD8+ T-cells and an increase in T_{EM} and T_{EMRA} CD8+ T-cells. However, when data were analyzed for absolute numbers, a significant decrease in T_N and T_{CM} a significant increase in T_{EMRA} CD8+ T-cells was observed (Gupta 2005). Fagnoni et al (2002) also observed an increase in primed CD8+CD28-CD45RA+ (equivalent to T_{EMRA}) in aged humans.

6 Apoptosis of Naïve, Central Memory and Effector Memory T-Cell Subsets in Aging

Herndon et al. (Herndon et al. 1997) have provided evidence of increased AICD of naïve T (CD45RO-) cells in aged humans and suggested its role in age-associated T-cell deficiency. However, they did not investigate apoptosis in memory T-cells. Brezinska et al. (2004) concluded that AICD (as measured by DNA content and caspasese-3 activation) in CD8+CD28+ (containing T_N and T_{CM}) and CD8+CD28- (containing T_{EM} and T_{EMRA}) was comparable. However, these investigators presented data from a single middle aged individual.

We have reported that in aged humans, both CD45RA+ (naïve) and CD45RO+ (memory) CD4+ and CD8+ T-cells were more sensitive to anti-CD95-induced apoptosis as compared to young subjects (Brzezinska et al. 2004). Furthermore, CD45RO+ displayed greater sensitivity to anti-CD95-induced apoptosis as compared to CD45RA+ CD4+ and CD8+ T-cells in both young and aged subjects. Miyawaki et al. (1992) also reported that healthy adult memory T-cells are more susceptible to anti-CD95-induced apoptosis as compared to naïve T-cells. We reported decreased expression of Bcl-2 in both CD4+ and CD8+ T-cells from aged humans as compared to young subjects; however, we did not examine Bcl-2 expression in naïve and memory subsets (Aggarwal and Gupta 1998). Shinohara et al. (1995) demonstrated decreased Bcl-2 expression in memory subsets of CD4+ and CD8+ T-cells in healthy adults. This would be consistent with our observation of increased sensitivity of memory T-cell subsets to death-receptor-mediated apoptosis as compared to naïve T-cell subsets. Although a role of Bcl-2 family protein in death

receptor pathway has been argued, Iwai et al. (1994) and Yoshina et al. (1994) have demonstrated that Bcl-2 blocks anti-CD95-induced apoptosis in mitogen-activated T-cells. Therefore, it is likely that decreased Bcl-2 expression in aging may play a role in increased sensitivity of T-cell subsets in aged humans. However, experiments of Bcl-2 overexpression need to performed to define a definitive role of decreased Bcl-2 in increased susceptibility of aged naïve and memory subsets to death-receptor-mediated apoptosis.

We have also examined TNF-α-induced apoptosis in both naïve and memory subsets of CD4+ and CD8+ T-cells, using TUNEL assay and flow cytometry and observed that both CD45RA+ naïve and CD45RA- memory CD4+ and CD8+ T-cells from aged individuals were more sensitive to TNF-α-induced apoptosis (Aggarwal and Gupta 1998).

As discussed above, naïve T-cells, as defined by the presence of CD45RA contain T_{EMRA} CD8+ T-cells (and a very small population of T_{EMRA} CD4+ T-cells), and CD45RA- (CD45RO+) contain both T_{CM} and T_{EM} CD8+ T-cells. Therefore, we have examined the relative sensitivity of T_N and T_{CM}, T_{EM} and T_{EMRA} CD8+ and CD4+ T-cell subsets to TNF-α-induced apoptosis. Naïve CD8+ T-cells and central memory CD8+ T-cells are more sensitive to death-receptor and oxidative stress-induced apoptosis, whereas effector memory CD8+ T-cells are resistant to apoptosis (manuscript in preparation). In aged humans we observed that naïve and central memory CD8+ T-cells displayed increased TNF-α-induced apoptosis as compared to young subjects, which is associated with increased caspase-8 and caspase-3 activation. In contrast, effector memory subsets are resistant to TNF-α-induced apoptosis and display minimal caspase activation in both young and aged subjects. Therefore, it appears that during aging decrease in naïve CD8+ T-cells is due to both decreased thymic output as well as increased apoptosis. We have also observed increased apoptosis in T_N and T_{CM} (T_{CM}.> T_N) CD4+ T-cells in aged humans as compared to young subjects; however, no significant difference was observed in the apoptosis of T_{EM} and T_{EMRA} CD4+ T-cells between aged and young humans; both were resistant to apoptosis (Gupta and Gollapudi 2006).

There are several possible mechanisms to explain differential sensitivity of various memory subsets to apoptosis. Since T_{CM} cells have high a replicative property (more than T_N cells), increased apoptosis may be critical to make niche for new T_{CM} CD4+ and CD8+ T-cells and to maintain homeostasis of T_{CM} cells. It is known that IL-7 and IL-15 provide survival signals in maintaining memory T-cells (Schluns and Defracois 2003; Alpdogan and van den Brink 2005). Furthermore, we have observed that IL-7 serves as an important preferential survival factor for T_{CM} cells, whereas, IL-15 provides preferential survival signal for T_{EM} and T_{EMRA} T-cells (Personal unpublished observations). A decreased IL-7 in aging may contribute to increased apoptosis of T_{CM} cells in aging. The low replicative property of T_{EM} and T_{EMRA} cells, which does not allow for the creation of an "immunological nitch" may be responsible for relative resistance of T_{EM} and T_{EMRA} cells to apoptosis. A large number of studies have been reported on CD8+CD28- T-cells generated after repeated stimuli (as a model of aging) and reported features of replicative senescence (low proliferative potentials and resistance to apoptosis). However,

Brzezinska et al. (2004) have reported that aged CD8+CD28- proliferate more than adult counterparts. We have observed that both T_{EM} and T_{EMRA} CD8+ T-cells from young and aged subjects can proliferate well in the presence of exogenous IL-2 and IL-15 (unpublished observation). We have also observed increased expression of IL-15 gene in CD8+ T-cells from aged humans (by gene array). These observations suggest that CD8+CD28- T-cells generated by repeated activation in vitro are not a true model for CD8+CD28- T-cells in aged humans. Furthermore, increased accumulation of CD8+CD28- T-cell population in aged humans may be due to an increased growth provided by increased IL-15 in aging.

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Mitochondria

Mitochondria and Immunosenescence

Pazit Beckerman and Arie Ben Yehuda

Contents

1 Introduction

The immune system undergoes age-associated changes, that affect its response to infections and cancer, and contributes to the organism's aging and its associated pathologies. An eminent hypothesis to explain the aging process, most supported by experimental data, is the mitochondrial free radical theory. Evidence is accumulating, linking mitochondrial oxidative damage and apoptosis to immunosenescence.

2 Mitochondria- Structure and Biology

Mitochondria are ubiquitous organelles that are intimately involved in many cellular processes. Its principal task is to provide the energy necessary for normal cell functioning and maintenance. Mitochondria are composed of several compartments, each with specific metabolic functions, including the inner and the outer membranes, the intermembrane space and the matrix. The inner membrane joins the mitochondrial cristae at specific junctions. The cristae contain the electron transport chain (ETC), phosphorylation apparatus, and membrane transporters [1].

The electron donors, NADH and $FADH_2$, provide reducing equivalents to the ETC. The ETC is composed of 4 multisubunit enzyme complexes. NADH is oxidized by complex I, reducing the lipid soluble mobile electron carrier coenzyme Q. Complex III oxidizes reduced coenzyme Q and in turn reduces the mobile carrier protein cytochrome c that donates its electron to cytochrome oxidase, complex IV, for the reduction of oxygen to water. The complexes of the ETC are likely to

P. Beckerman (✉) · A. B. Yehuda
University Hospital Kerem
The Department of Medicine
Hadassah Ein

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be organized in larger supercomplexes, forming a respirasome, in order to optimize channeling of reducing equivalents. Electron transfer down the redox potential gradient is coupled to the active transport of hydrogen ions from the matrix to the cytosol. This process requires a tightly controlled permeability of the inner membrane to ions and small molecules.

The phosphorylation apparatus uses the inner membrane proton gradient to phosphorylate ADP by complex V. Complex V couples proton flow down the electrochemical gradient from outer aspect of the inner membrane to the matrix side and the energy is utilized to drive complex V resulting in ATP production.

Uncoupling of respiration from ADP phosphorylation is a mechanism of physiologic regulation of the rate of oxidative phosphorylation. Uncoupling of respiration in pathologic states occurs due to damage to either the integrity of the inner membrane or of complex V. Uncoupling of respiration in pathologic states is more likely to collapse the electrochemical gradient, impairing energy production and increasing the probability of mitochondrial permeability transition [1, 2].

3 Mitochondrial Diseases

The classic mitochondrial diseases result from mutations in mitochondrial DNA (mtDNA) or nuclear genes that disrupt mitochondrial respiratory functions. These disorders have brain and skeletal muscle manifestations, and are often referred to as mitochondrial encephalomyopathies. Hundreds of point mutations, deletions and rearrangements have been associated with these diseases. Since all of the mitochondrial diseases have a disrupted respiratory function, one might expect a similar phenotype. The clinical variability is large, however, with many disease exhibiting tissue specific manifestations.

The role of mitochondrial dysfunction in a number of common conditions including the process of aging is being slowly elucidated. Evidence is emerging to suggest that mitochondria play a key role in the etiology of neurological disorders.

Parkinson's disease is a chronic neurodegenerative condition. Mitochondrial dysfunction, and in particular oxidative stress, has been implicated in its pathogenesis. Deficiencies of complex I have been observed in some patients with Parkinson's disease [3, 4], in the substantia nigra and subsequently in the peripheral tissues. Complex I is the target of toxins known to produce parkinsonian features in humans, such as MPTP. Inhibition of complex I results in increased free radical generation and could contribute to the oxidative mediated damage seen in Parkinson's disease. Families with mtDNA mutations and Parkinsonism have been identified [5]. Several studies that have sequenced mtDNA in Parkinson's disease patients have not identified any consistent mutations, although none has focused on Parkinson's patients with complex I deficiency. Mitochondrial complex I deficiencies have been described not only in the brain but also in skeletal muscle, platelets and lymphocytes in Parkinson's disease [6]. The relation between mitochondrial dysfunction and Parkinson's disease has evoked an attempt to develop treatment

that might improve disease progression, using coenzyme Q_{10}, that may enhance respiratory chain function [7].

Mutations in the genes for amyloid precursor protein are associated with Alzheimer's disease. Amyloid Aβ can inhibit oxidative phosphorylation in mitochondria [8]; impaired COX activity, reduced immunoreactive protein or decreased mRNA for mtDNA encoded proteins have been observed in the Alzheimer's disease brain; Aβ directly interacts with a mitochondrial enzyme, ABAD. ABAD is important in cell function, its inactivation results in a lethal phenotype, it is up regulated in Alzheimer's disease neurons and its coexpression with amyloid precursor protein exacerbates Aβ induced free radical mediated cell damage and death [9]. ABAD and Aβ colocalize in the mitochondria of Alzheimer's disease cortex and this interaction causes increased mitochondrial activity and apoptosis.

4 Mitochondria and Aging

The mitochondrial theory of aging states, that the original insult to mtDNA is induced by the continuous generation of reactive oxygen species (ROS) and other toxic species. mtDNA may be particularly susceptible to oxidative damage due to its lack of protective histones and its proximity to the inner mitochondrial membrane, where reactive oxygen species are produced. Some of the mutations in mtDNA impair respiratory chain function, leading to increased ROS production and further mtDNA damage, creating a vicious cycle. The positive feedback between mtDNA mutation and generation of ROS is thought to result in an increase in oxidative damage during aging, with eventual loss of cellular and tissue functions through a combination of energy insufficiency, signaling defects, apoptosis and replicative senescence.

Two mechanisms combine to increase ROS production from mitochondria in aged tissues. First, decreased flux through the electron transport chain increases the reduction of upstream complexes, especially complexes I and III, enhancing electron leak that generates ROS. Second, aging-induced modification of individual electron transport chain complexes can directly result in a greater fraction of electron leak.

Decreases in enzyme activity of an ETC complex can lower the rate of oxidative phosphorylation [1]. A decrease of 30-50% in activity is probably needed to result in a significant maximal rate lowering. The sites of greatest control of respiration are complex I in the ETC and the adenine nucleotide translocase and complex V in the phosphorylation apparatus. These sites require the least decrease in enzyme activity for decreases in the rate of oxidative phosphorylation to occur. Aging may alter the inner membrane, thereby impairing the activity of ETC complexes, or alter complex V, thereby slowing the rate or efficiency of phosphorylation.

Mitochondrial transmembrane potential is the driving force of cellular ATP formation, and its reduction can lead to ATP depletion and cell deenergization. Evidence show that oxidants may induce mitochondrial transmembrane potential reduction and

mitochondrial depolarization by promoting mitochondrial permeability transition due to oxidation of mitochondrial pyridine nucleotides and glutathione [10].

In support of this hypothesis many studies have linked ROS production and oxidative stress to aging and longevity [11].

Many tissues from aged individuals have lower respiratory function compared to tissues from younger individuals. Many reports also demonstrate that the rate of production of ROS from mitochondria increases with age in mammalian tissues: an increase of ROS was found in hepatocytes from aged rats [12], higher levels of peroxide and increased peroxide production after an adriamycin-induced oxidative stress. ROS production was also shown to increase in senescent fibroblasts and aging skeletal muscle cells.

A strong negative correlation has been demonstrated between expected lifespan and metabolic rate and ROS production rate of different species [13], and between lifespan and membrane lipid saturation.

Interventions and mutations that prolong survival tend to decrease the production of ROS from mitochondria, providing further evidence to the connection between aging and mitochondrial function: Calorie restriction has been shown to extend longevity; it increases the life span of rodents and delayed autoimmunity and onset of malignancy in mice. Calorie restriction also reduces the over-production of various T-cells subsets while maintaining the capacity of cells to respond to mitogens. It also maintains appropriate levels of apoptosis, including responses to dexamethasone induced death. The potency of NK and cytolytic T-cells was also maintained for longer periods in calorie restricted mice [14]. Sohal et al. proposed that calorie restriction significantly reduces aging of the mitochondria and production of ROS [15]. It attenuates age related changes in lipid peroxidation. It also decreases mitochondrial ROS production at complex I and lowers mtDNA oxidative damage. The major impact of calorie restriction on mitochondrial respiration appears to be a modulation of state 4 respiration, which increases via an increase in uncoupling protein content. The decreased coupling of respiration results in a decreased production of ROS that is reversed by an increase in fat intake (reviewed in 1). Calorie restriction also seems to trigger an adaptive response protecting the most basic requirements of membrane integrity.

Antioxidants experimental effect on aging may also demonstrate the mitochondrial relation, since it has been suggested that improvement in the age-related decreases in mitochondrial oxidative phosphorylation caused by antioxidants, attenuates aging. Still, the role of antioxidants in longevity is disputed. Studies comparing constitutive antioxidant levels between mammalian species, and experiments increasing or decreasing their tissue antioxidant concentrations in different ways, indicate that antioxidants do not seem to control aging rate, although they can protect against different pathologies and early death (reviewed in 16).

ROS have been shown to target al.l biomolecules in the cell, which undergo chemical modifications that accumulate with age- protein carbonylation and methionine oxidation, advanced glycation end-products, lipid peroxidation and nucleotide modifications [11].

Finally, mtDNA point mutations and deletions are more prevalent in aged tissues and cells. Numerous studies have documented the presence of large mtDNA deletions from muscle and brain from old individuals. A minimal threshold level of 90-95% of mutated mtDNA is usually necessary to impair respiratory chain function, depending on the type of mutation and the tissue affected. This may result from extensive fragmentation of mtDNA in minicircles in elderly subjects, increasing the amount of mtDNA mutations [17].

Two mouse models have further implicated mtDNA mutations in the aging process. Knockin mice have been developed by two research groups [18, 19]. These mice express a proofreading-deficient PolgA, the nuclear encoded catalytic subunit of the mtDNA polymerase, and acquire mtDNA mutations at a much higher rate than normal. The PolgA mutator mice accumulate mtDNA mutations in numerous tissues, reproducing the effect of aging. The mice have a phenotype consistent with premature aging, including osteoporosis, reduced activity, alopecia, reduced fertility, cardiac hypertrophy, and severe weight loss with decreased muscle mass and lipoatrophy. Interestingly, in spite of the widespread mutations, these mice do not appear to have any change in the levels of hydrogen peroxide or increased oxidative damage to DNA, proteins or lipids [19]. Still, evidence shows that mitochondrial but not cytosolic targeting of catalase, an antioxidant enzyme, over-expression enhances lifespan and reduces age-related cardiac pathology and cataracts. This further emphasizes the contribution of the mitochondrion to free radical mediated cellular damage and dysfunction in relation to aging [20].

Many tissues in error-prone PolgA mice described above contain increased levels of caspase-3. This increase in caspase-3 activation was also observed in tissues from normal aged mice. The mutant mice also showed increased TUNEL staining, an indication of the DNA fragmentation, a hallmark for apoptosis. Therefore, the diverse signs of aging in these mice may be due to apoptosis induction [19]. The induction of apoptosis may be related to the observation that patients carrying high loads of certain mtDNA mutations show a high degree of TUNEL-positive muscle fibers. Widespread apoptosis is also found in mouse embryos lacking mitochondrial transcription factor A, which is necessary for mtDNA expression and maintenance. These studies support the hypothesis that mtDNA mutations accumulation can induce the premature emergence of aging associated features (reviewed in 21). The role of mtDNA mutations in normal aging still remains to be elucidated.

5 Mitochondria and Immunosenescence

The mechanisms involved in immunosenescence have not been fully deciphered yet. The mitochondria could contribute to alteration of the age-related immunodeficiency by two mechanisms. First, like any eukaryote cell, lymphocytes require oxidoreductase processes by mitochondria, via the respiratory chain. Second, mitochondria are involved in apoptosis, a major process in T-cell death.

Mitochondrial modifications in the immune system cells are still largely obscure. Peripheral lymphocytes of 366 healthy individuals were examined by electron microscope. Ultrastructural mitochondrial damages increased from 50 years of age until 80 years, but after 80 years decreased [22]. The morphologic changed consisted of disappearance of the mitochondrial cristae, which were replaced by a lamellar structure, electron dense and electron opaque material, that was similar to lipofuscin.

With increasing age, human lymphocytes express reduced proliferation in response to mitogens [23], suggesting that the mitogenic stimulus induces stress which is better tolerated by cells from young, rather than from old individuals. It has been shown that antioxidants were able to recover the age dependent impairment of lymphocyte response to mitogens [24], which suggests an oxidative stress.

A few studies have evaluated the age dependent alterations of mitochondrial parameters in immune cells.

A clear cut delay of the increase in ATP following phytohemagglutinin stimulation has been shown in older human cells [25]. Also, a decrease of mitochondrial respiration with aging of mouse splenic lymphocytes was found [26].

Several studies have identified, using fluorescent probes specific for mitochondrial transmembrane potential, a decrease in respiratory activity of murine lymphocytes during aging [27]. The existence and maintenance of the lymphocyte transmembrane potential involves two main mechanisms: the active transport of monovalent cations, sodium and potassium, and their diffusion through membrane pores. The reduction in ATP-ase activity during aging may lead to the reduction in membrane potential.

Another study used two mitochondrial specific probes with a potential dependent or independent uptake, and found that the decline in the respiratory activity in the mouse occurred approximately six months prior to the decrease in mitochondrial membrane mass [28]. Respiratory activity of splenocytes decreased with age in animals older than six months to 50% of its initial level by 24 months. Mitochondrial membrane mass decreased after 12 months, by 25% up to 24 months. These results, with minor differences, were repeated in rat cells [29], showing that respiratory activity per unit of mitochondrial mass declined in an age dependent manner.

Rottenberg et al. showed that spleen lymphocytes from old mice had lower respiration rates than lymphocytes from young mice. Cyclosporine, an inhibitor of the mitochondrial permeability transition (PT), restored normal respiration rates to lymphocytes from old mice, suggesting enhanced susceptibility to mitochondrial permeability transition activation. By using DiOC6 as a probe for mitochondrial transmembrane potential, they showed that lymphocytes from old mice also had a lower mitochondrial membrane potential than lymphocytes from young mice, which was also restored by cyclosporine. Lymphocytes from old mice also exhibited a more oxidized state, as represented by the ratio FAD/FADH, a useful measure for the redox potential in mitochondria. It was suggested that enhanced generation of ROS leads to increased oxidative stress in lymphocytes from old mice, which renders their mitochondria more susceptible to PT activation [30].

These results were later demonstrated on leukocytes from healthy human volunteers of different age groups. Leukocytes were subjected to oxidative injuries by exposure to t-butylhydroperoxide, and were labeled with fluorochromes for measuring mitochondrial transmembrane potential, membrane peroxidation and mitochondrial oxidant formation. Mitochondrial transmembrane potential declined after t-butylhydroperoxide exposure, and the change was more prominent in leukocytes from older individuals. Cyclosporine A partially restored mitochondrial transmembrane potential, implying again the contribution role of PT. The mitochondrial depolarization was accompanied by increased oxidant formation and oxidation of pyridine nucleotides, which were more prominent in older individuals [31].

Studies of age-induced immune dysfunction suggest that the decline of the immune system response is largely due to T-cells dysfunction, which is associated with shifts in the composition of the T-cells population, specifically, a shift from a low memory to naïve ratio to a higher ratio [32]. One of the dysfunctions identified in T-cells from old rodents and humans is an attenuation of calcium signaling, which accompanies the expansion of memory T-cells [33]. Mather et al. review the changes in calcium signaling in aging T-cells [34]. Activation of TCR receptor induces a sustained elevation of calcium ions, which activated the nuclear factor of activated T-cells, transcription factors, and initiates the transcription of genes of the immune response. Ionomycin, a calcium ionophore that induces a sustained increase in calcium ions, induces T-cells proliferation, but is much less effective in raising calcium levels in T-cells from old mice, suggesting that calcium signaling mechanisms might be modulated in aging. The small fraction of ionomycin resistant cells in T-cells preparations from young mice, similar to the majority of cells from old mice, consists of memory cells.

Thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} ATPase releases calcium from internal stores and activates calcium release activated calcium channels in T lymphocytes. The thapsigargin-induced sustained calcium elevation was shown to depend critically on mitochondrial calcium uptake, which is driven by the mitochondrial transmembrane potential. The mitochondria remove Ca^{2+} from the vicinity of the calcium channels, thus preventing their activation. Inhibition of mitochondrial calcium uptake also inhibits the T-cell receptor-induced sustained elevation of calcium. Permeability transition (PT) is a large non specific channel that is activated by calcium and ROS. Its activation collapses the mitochondrial transmembrane potential, inhibits oxidative phosphorylation and calcium sequestration by mitochondria, and may induce apoptosis. Mather et al. showed that in T lymphocytes from young mice, the ionomycin-induced elevation of cell free calcium was inhibited by collapsing the mitochondrial membrane potential by uncouplers and ionophores, and activation of the PT. In T lymphocytes from old mice, ionomycin is ineffective in sustaining the calcium elevation, but treatment with cyclosporine A, which inhibits PT, restores the ionomycin-sustained calcium elevation. The enhanced activation of PT in T-cells from old mice was associated with enhanced oxidation of mitochondrial FAD, therefore aging may result in a reduc-

tion in mitochondrial transmembrane potential and enhanced oxidation of T-cell mitochondria, thereby activating PT and inhibiting calcium elevation, which affects T-cells proliferation [34].

Ayub et al. suggested that this change in calcium influx, whether mitochondria-mediated or not, is actually the result of activation of apoptotic pathways. Fas-stimulation of T-cells was shown to block calcium influx [35], a blockade that was specific for the fas-induced apoptosis route. A similar uncoupling of calcium influx from the calcium store release was observed in neutrophils [36]. The mechanism by which fas ligand uncouples calcium channel opening is not yet resolve, although the mechanism described above, including loss of mitochondrial membrane potential, is a possible explanation [37].

A number of studies measured the enzyme activity of individual electron transport chain complexes or oxygen consumption by leukocytes as an index of aging-related decrease in oxidative function of these cells.

Drouet et al. examined oxidative phosphorylation parameters with aging in lymphocytes [38]. Lymphocytes were retrieved from human volunteers, aged from 23 to 98 years, who were divided into two age groups, with average ages of 35 and 80.8 years. T-cells subpopulation analysis revealed a decline in absolute count of naïve cells in the elderly, whereas no significant change was observed in the percentage and absolute number of memory cells. Activity of complexes II, III, and IV of the respiratory chain was analyzed. Complex III activity did not change with aging, however, a significant decrease in complex II+III activity occurred in the elderly group. No difference was observed in complex IV activity between the groups.

The authors suggested that decline in complex II activity with aging could be secondary to a decline in the levels of active enzyme molecules per mitochondrion, or due to accumulation of altered molecules in the organelle. The decreased production of energy in the mitochondria, together with an increase of oxidative stress with aging, can activate the mitochondrial permeability transition pore and initiate apoptosis.

The sensitivity of lymphocytes in this study to specific inhibitors of respiratory chain complexes, such as rotenone and malonate, was high and unaffected by age, as opposed to an effect previously shown on human platelets.

Drouet et al. also examined the possibility that the decreases in respiratory chain activity could be secondary to mtDNA mutations, but found no mtDNA deletion, concluding that the dysfunction could be related to nuclear DNA damage, a suggestion that requires more investigation.

Sandhu et al. measured the activities of complexes I-V and CS [citrate synthase] in crude mitochondria fraction from four brain areas as well as from lymphocytes, from 1, 3-4, 12 and 24-month-old age group rats [39]. Age related alterations in mitochondrial electron transport chain complexes I-V and CS were observed. With the increasing age of the rats, a significant decline was seen in the specific activity of complexes I-V and CS. Since mtDNA encodes seven subunits of complex I and three subunits of complex IV, the authors suggest that the pattern of complex I and IV activities may be consistent with mtDNA deletions.

This study also correlated mitochondrial dysfunction with simultaneous aging in brain and immune cells. Interestingly, T-cells mediated immunity dysfunction has been implicated in the etiology of many of the chronic neurodegenerative diseases in the elderly. Several studies also give partial indication that lymphocyte analysis may provide an easy noninvasive method for investigating respiratory chain enzymes and assessing mitochondrial function in patients with neurodegenerative diseases.

6 Mitochondria and Apoptosis

Mitochondria play a central role in the regulation of programmed cell death.

Cells undergoing apoptosis exhibit a decrease in mitochondrial transmembrane potential that precedes nuclear signs of apoptosis. This applies to different cells types, including lymphocytes exposed to glucocorticoids or other lethal activation signals (40, 41). Apoptosis induced by pathologic stimuli is preceded by mitochondrial transmembrane potential dissipation. Both transcription of mitochondrial genome and synthesis of mitochondrial proteins are perturbed early during the apoptotic process. Loss of mitochondrial function is also observed in anucleate cells induced to undergo apoptosis, indicating that apoptotic alterations of mitochondrial function can occur in complete independence of the nucleus. Mitochondria are required in some cell free systems to induce nuclear apoptosis. Cyclosporine A, a PT inhibitor, efficiently prevents the apoptosis associated fall in mitochondrial transmembrane potential, which may indicate that apoptotic mitochondrial transmembrane potential reduction results from PT.

Direct induction of PT by protoporphyrin IX, which is well known for its PT-triggering capacity, induced mitochondrial transmembrane potential disruption, enhanced generation of superoxide anions, and increased signs of apoptosis in thymocytes and T-cells from mice, as evidenced by DNA hypoploidy and fragmentation and chromatin loss [42].

Regulation of T-cells apoptosis is essential for lymphocyte homeostasis and immune functions [43]. During an adaptive immune response naïve and memory T-cells proliferate and fulfill their effector function. This expansion phase is followed by the contraction phase, in which T-cells numbers decline and reach normal levels. This process is highly regulated and requires a switch from an apoptosis resistant towards an apoptosis sensitive state in T lymphocytes. T-cells homeostasis is basically controlled by two separate apoptosis pathways: activation induced cell death (AICD) and activated T-cells autonomous death (ACAD). In ACAD, cell death is determined by the ratio between anti- and pro-apoptotic Bcl-2 family members at the mitochondria. The intrinsic cell death pathway critically depends on permeabilization of the outer mitochondrial membrane for cell death execution. A number of apoptotic signals converge on mitochondria, such as oligomerization of the apoptotic Bax and Bak proteins, leading to permeabilization of the outer membrane and

release of cytochrome c, apoptosis inducing factors and Smac/DIABLO into the cytoplasm. Cytochrome c binds to adaptor molecules, including apoptosis protease activating factor 1 (Apaf-1) and initiator pro-caspase proteins, forming an 'apoptosome', which leads to cleavage of pro-caspase-9 to active caspase 9, which can then activate downstream effector caspases 3 and 7, resulting in apoptosis. During ACAD, the pro-apoptotic Bcl-2 and Bcl-X_L are found in a constitutive association with Bim on the mitochondrial membrane, blocking the apoptotic function of Bim assuring cell survival. The ratio between Bcl-2 versus Bim regulates T-cells death. Mitochondria in apoptotic cells are also believed by some to release considerably more ROS.

7 Apoptosis and Immunosenescence

Apoptosis plays a key role in a variety of immune processes, including elimination of potential anti-self clones, removal of faulty pre-B and pre-T-cells arising in the marrow and thymus, and also destruction of virally infected and tumorigenic cells by natural killer cells and cytolytic T-cells. Given the importance of apoptosis in the normal functioning of the immune system, immunosenescence itself could be altered by age related changes in apoptosis, including the mitochondrial pathway. Controversial data exist in the literature. Some investigators have reported a decrease of Fas/FasL-induced apoptosis in aged animals, and in human CD8+ T-cells reaching replicative senescence after multiple rounds of antigen-specific proliferation [44].

Increased resistance to apoptosis was found in cells from people chronically exposed to oxidative stress, as in patients affected by Fanconi's Anemia or uremia.

Monti et al. [45] examined peripheral blood mononuclear cells from three age groups of human donors. They induced apoptosis by 2-deoxy-D-ribose (dRib), an agent that induces apoptosis in mononuclear cells by interfering with cell redox status and mitochondrial membrane potential. They found an inverse correlation between the age of the donors and the propensity of their mononuclear cells to undergo apoptosis. Cells from old people showed an increased resistance to dRib-induced glutathione depletion and a decreased tendency to lose the mitochondrial membrane potential. No difference in Bcl-2 was found. This indicates a decreased tendency to undergo apoptosis in the old. Moreover, the increased resistance of dRib-induced apoptosis of mononuclear cells appeared to be related to glutathione depletion, but independent of Bcl-2 content, suggesting mitochondrial involvement that is age related.

On the other hand, others identified increased apoptosis in lymphocytes from elderly people following activation with anti-CD3, Phytohemagglutinin, Concanavalin, or activation with polyclonal mitogen plus anti-Fas treatment.

Gupta et al. [46] examined T-cells subsets of the aged (65-95 years) and the young (20-29 years) using peripheral blood T-cells. They found increased expression of Fas and FasL on both CD4+ and CD8+ lymphocytes of the aged subjects.

They also compared naïve and memory cells. A decreased expression of Bcl-2 (anti apoptotic protein) and increased expression of Bax (pro apoptotic protein) was noted in naïve and memory T-cells of the aged [47-48].

Monti et al. [45] suggested that this apparent contradiction in results can be explained taking into account the experimental setting, and hypothesized that aging is characterized on one hand by an increased tendency to undergo apoptosis in activated lymphocytes, and on the other hand, by a decreased tendency to undergo apoptosis as a more general process of senescence.

These changes in aging T-cells could explain the reduced number of naïve T-cells produced in the elderly, and contribute to the early termination of immune responses in the elderly.

Another explanation to the different response of memory versus naïve cells was offered by Kim et al. They have demonstrated that splenic T lymphocytes from old mice exhibit a significant decline in mitochondrial membrane potential; yet despite this change, there is a lower rate of withdrawal apoptosis in the memory $CD4^+$ and $CD8^+$ T-cells. To explain the survival of the cells in spite of increased oxidative stress, the authors demonstrated increased glutathione production and phase II enzyme (antioxidants) expression, which protect the memory T-cells. Phase II enzymes play a role during aging, and age-related changes in their expression were shown in various tissues, including brain and liver. Kim et al. showed similar increases in memory T-cells, compared to naive cells. Moreover, compared with wild type mice, mice lacking the expression of NF-E2-related factor-2, the transcription factor that regulated phase II enzyme expression, had a significantly increased rate of apoptosis in response to an oxidative stress stimuli. These cells exhibit a greater decline in mitochondrial membrane potential and increased ROS production. The authors claim that this mechanism could contribute in part to the accumulation of memory T-cells during aging [49].

In summary, the aging process affects the function of multiple cells and organs in the human organism. One theory that explains the aging process, and supported by experimental data, is the mitochondrial free radical theory. According to this theory, ROS accumulate in the mitochondria, causing an oxidative damage to mitochondrial DNA and to the mitochondrial respiratory chain function, thereby causing the decrease in cellular function. A large body of evidence exists that supports this theory in different tissues, including heart, skeletal muscle and brain. Data is accumulating that similar processes also take place in the immune system. Studies on immune cells from humans and animals have shown age-related decreases in mitochondrial respiration, mitochondrial transmembrane potential, and respiratory chain complexes activity. Moreover, mitochondria are also involved in the apoptotic process, which in itself plays an important role in T-cells regulation and homeostasis, and is essential for immune system function. Changes in apoptotic processes have been shown to occur in cells of the aging immune system, thereby further emphasizing mitochondrial role in immunosenescence. Further studies are needed in order to understand to what extent mitochondrial changes with age influence the dysregulation of both innate and cognate immunity and what are the clinical consequences of such changes.

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Proteasome

Proteasome Activity and Immunosenescence

Bertrand Friguet

Contents

Abbreviations

G6PDH	glucose-6-phosphate dehydrogenase
HNE	4-hydroxy-2-nonenal
ROS	reactive oxygen species
RNS	reactive nitrogen species

Abstract: The proteasome is the main proteolytic system implicated in the removal of oxidatively damaged proteins, general turnover of intracellular proteins and targeted degradation of proteins that have been marked by poly-ubiquitination. Impairment of proteasome function has been associated with cellular aging in a variety of tissues and cell types including lymphocytes, and is believed to contribute to the age-related accumulation of oxidized proteins due to their decreased elimination by the proteasomal pathway. This chapter first summarizes the most relevant features of the proteasomal system and then expands on the current knowledge on the impact of aging on proteasome structure and function, taking in account the fate of proteasome upon oxidative stress situations. Finally, the possible implication of age-related alterations of the proteasomal system in the process of immunosenescence is presented.

Keywords: Proteasome • Aging • Protein oxidation • Damaged protein degradation • Immunosenescence

B. Friguet (✉)
Université Pierre et Marie Curie, Paris 6, UMR 7079
Case Courrier 256, 4 Place Jussieu, 75252 Paris cedex 05, France
Tel./Fax: 33 (0) 1 44 27 82 34
E-mail: bertrand.friguet@snv.jussieu.fr

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1 Introduction

Aging is a complex process controlled by genetic and environmental factors, which is accompanied by the decline of different physiological functions of an organism during the last part of its life. Damage to macromolecules has been implicated in the cellular degeneration that occurs during aging and accumulation of oxidized proteins represents a hallmark of cellular aging (Beckman and Ames 1998; Berlett and Stadtman 1997). Indeed, proteins are targets for numerous posttranslational modifications such as oxidation, conjugation with lipid peroxidation products and glycoxidation, that have been shown to affect their biological function (Davies 1987, 1993; Stadtman 1990, 2006). In addition, calorie restriction, the only intervention known to slow-down aging, delays the age-related accumulation of oxidatively damaged proteins (Goto et al. 2002; Shibatani and Ward 1996) while long-lived transgenic animals were found to exhibit a decreased load of protein oxidative damage (Orr and Sohal 1994). Elimination of damaged protein and protein turnover is critical to preserve cell function and the main proteolytic system in charge of cytosolic protein degradation is the proteasome, a multicatalytic proteolytic complex that recognizes and selectively degrades oxidatively damaged and poly-ubiquitinated proteins (Coux et al. 1996; Davies 2001; Grune et al. 1997; Voges et al. 1999). Since accumulation of oxidized protein with age can be due to increased protein alteration, decreased elimination (i.e., repair and degradation) of oxidatively damaged protein or the combination of both phenomenoms, one of the hypothesis put forward to explain oxidized protein build-up is a decrease of proteasome activity with age (Friguet 2006; Friguet et al. 2000; Gaczynska et al. 2001; Keller et al. 2000a). In fact, age-related impairment of proteasome has been documented in a variety of organs, tissues and cell types, which appears to be the result of numerous factors including decreased proteasome components expression, alteration and/or replacement of proteasome subunits and formation of inhibitory elements such as oxidatively modified cross-linked proteins. Since both age-related accumulation of damaged proteins and impairment of proteasome have been documented in lymphocytes (Beregi et al. 1991; Poggioli et al. 2002; Ponnappan et al. 1999; Sell et al. 1998), alterations of proteasome structure and function may therefore directly contribute to the complex process of immunosenescence.

2 Proteasomes

2.1 20S Proteasome Structure and Proteolytic Activity

The 20S proteasome is a high molecular weight multicatalytic proteolytic complex found in Archaebacteria and Eukaryotes that is implicated in the degradation of most of the intracellular proteins including oxidized and poly-ubiquitinated proteins (Coux et al. 1996; Davies 2001; Grune et al. 1997; Voges et al. 1999). This complex that has been first observed in erythrocytes by Harris in 1968 (Harris 1968), is ubiquitous in eukaryotic cells, in which it can represent up 1% of total soluble proteins (Tanaka

et al. 1986). In mammalian cells, the proteasome constitutes the main nonlysosomal proteolytic system involved in protein degradation in the cytosol and in the nucleus. Besides acting as a housekeeping enzyme by eliminating abnormal and oxidized proteins, the proteasome is also implicated in a broad range of cellular functions through the selective degradation of ubiquitin-targeted regulatory proteins such as transcription factors, cyclins and rate-limiting enzymes in important metabolic pathways (Cie-chanover and Iwai 2004; Goldberg et al. 1997; King et al. 1996; Pajonk and McBride 2001). The 20S proteasome exhibits a cylinder shape of about 15 nm length and 11 nm diameter and is made up of four stacked rings of seven subunits classified as α or β subunits (Groll et al. 1997; Hegerl et al. 1991; Lowe et al. 1995). The seven α subunits form the apical rings of the complex while the seven β subunits form the inner rings and carry the catalytic activities. The two outer chambers are formed by the junction of one α ring and one β ring and the central catalytic chamber is made by the junction of the two β rings. The eukaryotic proteasome has only three catalytically active β subunits: β1 for the peptidyl glutamylpeptide hydrolase (or postacidic) activity, β2 for the trypsin-like activity and β5 for the chymotrypsin-like activity that cleave peptide bonds after an acidic, basic and hydrophobic aminoacid, respectively (Coux et al. 1996; Groll et al. 1997; Kisselev et al. 1999). The specificity pockets S1 have been described as positive, negative or neutral electrostatic potential surfaces (Borissenko and Groll 2007). Two copies of each subunit are present in the catalytic chamber that contains six active sites. These activities can be conveniently assayed using specific fluorogenic peptides. Proteasomes have the unique property to use a N-terminal threonine as a catalytic residue. A maturation step is needed to generate the N-terminal amino group which implicates intramolecular autolysis to remove the prosegments of the β-subunit precursors. The proteolytic mechanism was elucidated using crystal structures of yeast and bovine liver proteasomes (Groll et al. 1997; Unno et al. 2002) and site-directed mutagenesis (Ditzel et al. 1998; Groll et al. 1999). Interestingly, when cells are exposed to such stimuli as IFNγ, TNFα or LPS the subunit composition of the 20S proteasome is modified, as inducible homologous subunits are incorporated in the structure upon de novo synthesis: the iβ1, iβ2 and iβ5 subunits, respectively replace their β1, β2 and β5 constitutive counterparts to form the immunoproteasome (Fruh et al. 1994; Gaczynska et al. 1993; Tanaka 1994). Such replacement of proteasome subunits modify proteasome peptidase activities and lead to higher chymotrypsin-like and trypsin-like activities and lower peptidyl glutamylpeptide hydrolase activity, thus increasing production of peptides with higher affinity for MHC class I complex (Fruh et al. 1994; Gaczynska et al. 1993; Kloetzel 2004; Rivett and Hearn 2004; Rock and Goldberg 1999; Tanaka 1994).

2.2 20S Proteasome Inhibitors

The development of selective inhibitors of the proteasome has been very useful for deciphering the cellular functions of the proteasome. The majority of proteasome inhibitors are short peptides bearing a reactive group which creates a cova-

lent bond with the catalytic N-terminal threonine such as peptide aldehydes (MG 132, Braun et al. 2005; Groll et al. 1997), peptide boronates (MG 262, bortezomib or Velcade™, Adams et al. 1998; Adams and Kauffman 2004) and peptide vinyl sulfones (Bogyo et al. 1997; Borissenko and Groll 2007). Epoxomicin is a peptide epoxyketone that is a natural molecule isolated from the actinomycete strain Q996-17. Epoxomicin preferentially inhibits the chymotrypsin-like activity and is characterized by its unique specificity for the proteasome (Elofsson et al. 1999). The natural β lactone lactacystin (Streptomyces sp.) is a nonpeptidic molecule that form covalent acyl ester bond with the N-terminal threonine (Borissenko and Groll 2007; Fenteany et al. 1995). Lactacystin in itself is not active against the proteasome but its spontaneous hydrolysis generates clasto-lactacystin β lactone (omularide) which binds specifically to the β5 subunit and inhibits the chymotrypsin-like activity. Noncovalent inhibitors of the proteasome have been investigated less extensively. The anti HIV protease Ritonavir and benzylstatine derivatives have been shown to inhibit the proteasome non-covalently (Furet et al. 2004; Schmidtke et al. 1999). The cyclic tripeptide TMC-95A, which is a metabolite of Apiospora montagnei, is a very potent inhibitor of all three peptidase activities of the proteasome (Koguchi et al. 2000). Non covalent binding of TMC-95A with the proteasome active sites has been demontrated by X-ray analysis (Groll et al. 2001). Other molecules have also been described as proteasome inhibitors such as gliotoxin (Kroll et al. 1999), lipopeptides (Basse et al. 2006), bi- or multivalent molecules (Loidl et al. 1999), ajoene (Xu et al. 2004), arecoline derivatives (Marastoni et al. 2004) and epigallocatechin-3-gallate analogs (Wan et al. 2005).

2.3 20S Proteasome Regulators and the 26S Proteasome

The eukaryotic 20S proteasome cylinder is closed in its latent form and can be switched to an active form under certain experimental conditions such as heat treatment, addition of fatty acids or detergent at low concentration (Ando et al. 2004; Dahlmann et al. 1985). In addition, the opening of the α rings can be promoted upon binding to the 20S proteasome of regulatory complexes such as PA700 (19S) or PA28 (11S), Dahlmann 2005). The 26S proteasome results from the ATP-dependent association with PA700 or 19S regulator and is an essential component of the ubiquitin–proteasome degradation pathway of poly-ubiquitinated proteins. The axial channel of the 20S proteasome is gated by the Rpt2 subunit of PA700 while PA28 stimulates 20S proteasome peptidase activities and may facilitate product release in vivo (Kohler et al. 2001). 20S proteasome can bind one or two 19S regulators resulting the formation of either "single-capped" or "double-capped" 26S proteasome. In addition, hybrid proteasome containing one PA28 and one PA700 complex associated at both end of the 20S proteasome can be formed (Tanahashi et al. 2000). The association of PA28 to the 20S proteasome is ATP-independent and results in an increase of proteasome peptidase activities while it does not improve protein degradation (Dubiel et al. 1992; Ma et al. 1992; Whitby et al. 2000). As for the immunoproteasome

subunits, the expression of PA28 subunits is induced after treatment of cell by IFNγ. In the cytosol PA28 is composed of two types of subunits α and β of about 28 kDa forming hexa or heptameric rings α3β3 or α3β4 while in the nucleus PA28 is made of single type subunit γ (Ahn et al. 1995; Knowlton et al. 1997; Mott et al. 1994). The 19S regulator is composed of at least eigthteen subunits belonging to either the «lid» or the «base» of the complex. Six of the nine subunits of the base are ATPases exhibiting a chaperone-like activity and are believed to participate to the unfolding of the substrate protein prior to its entrance in the 20S proteasome catalytic chamber and its degradation (Braun et al. 1999; Hershko and Ciechanover 1998; Kloetzel 2001). The lid subunits are involved in the recognition of polyubiquitinated protein substrates and recycling the ubiquitin moiety through isopeptidase activity (Deveraux et al. 1994; Hershko and Ciechanover 1998). Polyubiquitination of a protein is a complex process that requires ATP and involves ubiquitin, a 76 amino acids protein, and three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin ligases), to ensure specific recognition of the protein substrate (Ciechanover and Iwai 2004; Finley et al. 2004; Weissman 2001).

3 Age-Associated Impairment of Proteasome Function

3.1 Accumulation of Oxidized Proteins

Protein are oxidized as a result of oxidative insult derived from the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), that includes superoxide anion, hydrogen peroxide, the hydroxyl radical, nitric oxide and peroxynitrite (Berlett and Stadtman 1997; Dean et al. 1997). These reactive species are produced in the cell during the aerobic metabolism at the level of organelles such as mitochondria and peroxisome and by other enzymatic or nonenzymatic pathways (Beckman and Ames 1998). Increased ROS production is also achieved during situations of oxidative stress such as UV irradiation or inflammation. Basal production of ROS is part of normal cellular redox homeostasis, and antioxidants (enzymatic and nonenzymatic) regulate their level. However, when the balance between ROS production and elimination is disrupted, increased damage to macromolecules (including lipids, nucleic acids and proteins) occurs, leading to both reversible and irreversible oxidative modifications (Hensley and Floyd 2002). Within a protein, all amino acids can be oxidized but sulfur-containing (cysteine and methionine) and aromatic (tryptophane and tyrosine) amino acids are the most susceptible to oxidation (Stadtman and Levine 2003). In addition, tyrosine is a target for the reactive nitrogen species peroxynitrite, giving rise to nitrotyrosine. Oxidation of cysteine leads to disulfide bridges, mixed disulfides and cysteic acids, i.e. cysteine sulfenic, sulfinic and sulfonic acids (Requena et al. 2003). Formation of disulfide bridges, mixed disulfides or cysteine sulfenic acids is reduced by the thioredoxin/thioredoxine reductase or the glutaredoxin/

glutathione/glutathione reductase systems (Holmgren 2000; Levine and Stadtman 2001; Poole et al. 2004). Oxidation of methionine into methionine sulfoxide can be reversed catalytically by the peptide methionine sulfoxide reductases system (Grimaud et al. 2001; Sharov et al. 1999).

Oxidation of other amino acids most often leads to the formation of hydroxyl and carbonyl derivatives. Thus, detection of protein-associated carbonyl groups has been widely used for assessing the extent of protein oxidation. Several methods aimed at quantitative measurement of carbonyl groups within proteins are based on their specific derivatization by 2, 4–dinitrophenylhydrazine and immunochemical detection of such derivatized carbonylated proteins can be achieved using an antidinitrophenyl antibody (Levine et al. 1994). Upon oxidation, proteins usually become less active, less thermostable and more hydrophobic (Davies 1987; Fisher and Stadtman 1992; Friguet et al. 1994b). Protein damage can also originate from oxidation-derived reactions of amino acids such as lysine, arginine, histine and cysteine with lipid peroxidation products (e.g., 4-hydroxy-2-nonenal, malondialdehyde) or with sugars or derived metabolites to form glycoxidation adducts (e.g., carboxymethyl lysine, pentosidine). The resulting adducts that are formed on the protein often bring in carbonyl groups and/or cross-links (Friguet and Szweda 1997; Szweda et al. 2002). The function of these modified proteins is generally impaired or even completely inactivated.

Age-related increases in protein carbonyl content, taken as a signature of oxidative modifications, have been widely documented in different tissues and organisms: human dermal fibroblasts, human epidermal cells, human lenses, human erythrocytes, human brain, rat hepatocytes and whole *Drosophila* (Levine and Stadtman 2001; Petropoulos et al. 2000). In human keratinocytes and lenses, we have shown that increased protein oxidation is associated with increased protein glycoxidation and conjugation by lipid peroxidation adducts (Petropoulos et al. 2000; Viteri et al. 2004). Such an increase in oxidatively damaged protein is believed to affect cellular integrity, since it is associated with the impairment of key enzymes. Recent data argue for an age-related increase in protein carbonyl content such that elderly individuals would have one-third of their proteins in average carrying this oxidative modification (Stadtman and Levine 2000). However, not all proteins are equally sensitive to oxidation and there are growing evidence that only a restricted set of proteins would be preferentially affected. Indeed, Sohal and colleagues have already reported that in aging flies, two mitochondrial enzymes, aconitase and adenine nucleotide translocase, are specific targets for oxidative modification (Yan et al. 1997; Yan and Sohal 1998) and we have recently shown that glutamate dehydrogenase and ornithine carbamoyl transferase are preferentially glycoxidized in the liver mitochondrial matrix of old rats (Hamelin et al. 2007). Moreover, based on a proteomic approach, we have previously reported that age-related increases in protein glycoxidation and modification by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) are also restricted to preferential target proteins in human peripheral blood lymphocytes (Poggioli et al. 2002, 2004).

3.2 Oxidized Protein Elimination

Since repair of oxidized proteins is limited to reduction in specific oxidation products of sulfur-containing amino acids, oxidation of all other amino acids within a protein will target it for degradation (Carrard et al. 2002; Friguet 2006). Upon mild oxidation, proteins become more prone to proteolysis, while highly oxidized proteins usually become resistant to proteolysis because of the formation of intra- and intermolecular cross-links (Friguet and Szweda 1997; Grune et al. 1997). Oxidized proteins represent good substrates for degradation by the proteasome in vitro, and oxidized proteins have been shown to be preferentially degraded by the 20S proteasome in an ATP- and ubiquitin-independent manner in a variety of cell types (Davies 2001; Shringarpure et al. 2003). However, certain studies have reported that the ubiquitin-26S proteasome pathway is involved in degradation of oxidized protein from lens cells (Shang et al. 2001). Moreover, ubiquitination of proteins carrying glycoxidation and lipid peroxidation adducts has also been documented (Bulteau et al. 2001b; Marques et al. 2004). The increased susceptibility of an oxidized protein to proteolysis has been correlated with exposure of hydrophobic amino acids at the surface of the protein that may represent a recognition signal for degradation by the 20S proteasome (Davies 2001; Grune et al. 1997). Alternatively, such exposure of residues that are normally hidden in the hydrophobic core of the protein may result from decreased thermodynamic stability of the oxidized protein that renders it more flexible, especially at the C-terminus and/or N-terminus end of the protein, hence making it more prone to progressive degradation by either the 20S or 26S proteasomes (Goldberg et al. 1997). Interestingly, recent evidence has been provided that chaperone-mediated autophagy of proteins carrying a KFERQ motif is activated upon oxidative stress, implying participation of this proteolytic pathway in elimination of some oxidized proteins (Kiffin et al. 2004). Moreover, it has been also recently reported that when proteasome capacity is exceeded, autophagin expression is induced suggesting a physiological link between the lysosomal and proteasomal degradation systems (Klionsky 2005).

The proteasome appears to be a key actor in damaged protein elimination and other regulatory processes, and oxidative damage to protein has been implicated in age- and disease-related impairment of cellular functions. Therefore, the fate of the proteasome during oxidative stress has received particular attention. Indeed, peptidyl glutamyl peptide hydrolase and trypsin-like activities are readily inactivated upon exposure of the 20S proteasome to metal-catalyzed oxidation in vitro (Conconi et al. 1996, 1998). However, these alterations depend on whether the proteasome is in its active or latent state, a finding that may be related to the differential susceptibility to oxidative stress of the 26S and 20S proteasomes (Reinheckel et al. 1998). Moreover, in vitro treatment of the 20S and 26S proteasomes with nitric oxide or HNE was found to inactivate certain peptidase activities (Conconi and Friguet 1997; Farout et al. 2006; Ferrington and Kapphahn 2004; Glockzin et al. 1999). In addition, the proteasome is a target for modifications by oxidative proc-

esses in vivo that can lead to either its transient or irreversible inactivation. We first reported that FAO hepatoma cells, treated with iron and ascorbate in order to promote metal-catalyzed oxidation, exhibited decreased peptidyl glutamyl peptide hydrolase and trypsin-like activities, indicating that the proteasome was a target for inactivation upon oxidative stress (Conconi et al. 1998). Interestingly, both α-crystallin and Hsp 90 were found to protect proteasomes against oxidative insults in vitro, while depletion or overexpression of Hsp 90 in FAO cells resulted in decreased or increased protection of proteasome trypsin-like activity, respectively. This chaperone-mediated protection of proteasome activity during oxidative stress may be related to other antioxidant properties described, especially for small heat shock proteins (Arrigo 2001). In addition, neural SH-SY5Y cells stably transfected with human HDJ-1, a member of the HSP40 family, were shown to retain a greater preservation of proteasome activity following oxidative injury (Ding and Keller 2001). Taken together the data suggest that heat shock proteins may confer resistance to oxidative stress, at least in part, by preserving proteasome function. Oxidative stress induced in vivo by treatment with ferric nitriloacetate in kidney and ischemia-reperfusion in brain induced impairment of proteasome function correlated with the appearance of HNE-modified proteasomes (Keller et al. 2000c; Okada et al. 1999). Upon coronary occlusion-reperfusion, inactivation of trypsin-like activity has been associated with specific modification by HNE of three proteasome subunits (Bulteau et al. 2001a). In contrast, upon UV irradiation of cultured keratinocytes leading to a decline in proteasome activity, no modification of the proteasome was observed when the proteasome was purified from irradiated cells (Bulteau et al. 2002a). Proteasome inhibition resulted from the UV-induced increased load of damaged proteins, such as HNE modified proteins. In neural cells, inhibition of mitochondrial complex I by rotenone and 1-methyl-4-phenylpyridinium was found to increase the production of ROS and to inactivate the proteasome, most likely through oxidative damage and ATP depletion (Hoglinger et al. 2003; Shamoto-Nagai et al. 2003). Upon treatment of neuroblastoma cells with rotenone, a drastic reduction in proteasome activity was observed and suggested to originate from direct modification of 20S proteasome subunits by acrolein while aggregated acrolein-modified proteins coimmunoprecipated with the proteasome (Shamoto-Nagai et al. 2003). Conversely, proteasome inhibition has been shown to decrease complex I and complex II activities and to increase oxygen free radical production, indicating that mitochondrial homeostasis is altered, oxidative stress is triggered, and cell vulnerability to oxygen free radicals is increased as a result of proteasome inhibition (Hoglinger et al. 2003; Sullivan et al. 2004). These findings underscore the critical importance of the interplay of the different protein maintenance systems implicated in cellular redox homeostasis, protection against oxidative stress and oxidized protein removal. Finally, it has been recently shown that both 26S and 20S proteasomes peptidase activities could be inhibited upon treatment with the prooxidant buthionine sulfoximine of T cells from young donors resulting in an increase in oxidized proteins and a decline in both activation-induced proliferation and degradation of the NκB inhibitor, IκB (Das et al. 2007).

3.3 Decreased Proteasome Activity with Age

The age-related accumulation of oxidatively modified and ubiquitinated proteins, and the general decline in protein turnover, have raised the possibility that proteasome function is impaired with age (Carrard et al. 2002; Friguet et al. 2000). Pioneering studies from our group and that of Ward indicated that proteasome proteolytic activity is affected with aging (Conconi et al. 1996; Shibatani and Ward 1996; Shibatani et al. 1996). Indeed, we showed that the 20S proteasome from rat liver exhibited a 50% decrease for the peptidyl glutamylpeptide hydrolase activity when purified from old animals compared with young ones, while Ward and collaborators reported a 40% decrease in the same peptidase activity when activated by SDS and assayed in crude homogenates (Conconi et al. 1996; Shibatani et al. 1996; Shibatani and Ward 1996). Interestingly, we also reported that decreased protein uptake upon dietary self-selection of nutriments, can compensate for the age-related decrease of 20S proteasome activity observed with standard diet in rat liver (Anselmi et al. 1998). This finding may be related to the beneficial effects associated with dietary restrictions in calories and proteins, including decreased macromolecular damage, increased expression of antioxidant enzymes and increased longevity. Subsequently, we and other groups have reported that proteasome activity declines with age in a variety of tissues (Bardag-Gorce et al. 1999; Bulteau et al. 2002b; Hayashi and Goto 1998; Keller et al. 2000a; Merker et al. 2000; Petropoulos et al. 2000; Ponnappan et al. 1999; Viteri et al. 2004), although some studies have shown that this decline may not be universal. Such a decline in proteasome activity is believed to contribute to the age-associated build up of oxidized protein.

We have shown that the age-related decline in proteasome activity might be explained by decreased expression of proteasome subunits in human keratinocytes (Petropoulos et al. 2000), human fibroblasts (Chondrogianni et al. 2000), and rat cardiomyocytes (Bulteau et al. 2002b). Interestingly, fibroblasts from healthy centenarians exhibited proteasome activity and proteasome subunit expression levels closer to those of younger individuals than older ones, suggesting that sustained proteasome activity could have contributed to the successful aging of these individuals (Chondrogianni et al. 2000). In a more recent study, the exhaustive analysis of proteasome subunit expression in senescent WI 38 human fibroblasts has indicated that only the expression of catalytic β-subunits is decreased, and that less 20S proteasome is assembled while certain α-subunits are found in a free state (Chondrogianni et al. 2003). Moreover, exposure of WI 38 young fibroblasts to sublethal doses of the proteasome inhibitor epoxomycin resulted in a senescent-like phenotype. Transcriptome analysis using microarrays performed on both mouse skeletal muscle and human fibroblasts has shown decreased expression of several 20S and 26S proteasome subunits (Lee et al. 1999; Ly et al. 2000). In both analyses, performed with either post-mitotic or mitotic cell types, fewer than 2% of the genes monitored showed age-related altered expression, with very little overlap except for proteasome components. The gene expression profile observed with dietary-restricted old animals led the authors to propose that the anti-aging effect associated with dietary

restrictions may have originated from stimulation of protein turnover and decreased accumulation of macromolecular damage (Lee et al. 1999). Evidence for changes in the proteasome composition has been provided in certain age related neurodegenerative diseases (Vigouroux et al. 2004). Of particular interest is the Huntington disease where a concomitant increased of chymotrypsin-like and trypsin-like activities of the proteasome and an overexpression of the iβ1 and iβ5 inducible subunits were observed in the affected brain regions, indicating that changes in the 20S core particle subunit composition may play a role in neurodegeneration (Diaz-Hernandez et al. 2003, 2004). More recently Ferrington et al. reported in aged muscle a two to threefold increased of immunoproteasome whereas 20S proteasome expression was decreased. Moreover the low proteasome activity was attributed to a 75% reduced amount of PA700 and PA28 complexes, suggesting that in aged muscle, the endogenous content of proteasome activators is inadequate for complete activation of the 20S proteasome (Ferrington et al. 2005).

In addition to decreased and/or modified proteasome subunits expression, as an explanation for the age-related decline in proteasome activity, our initial finding of decreased peptidyl glutamylpeptide hydrolase specific activity of proteasome purified from aged rat liver was indicative of direct inactivation of the proteasome (Anselmi et al. 1998; Conconi et al. 1996). Further studies on proteasome purified from rat liver or cardiomyocytes and human epidermis showed decreased proteasome proteolytic activity coupled with subunit replacement and/or posttranslational modifications, as evidenced by comparison of two-dimensional gel electrophoretic patterns of proteasome subunits (Anselmi et al. 1998; Bulteau et al. 2000; Bulteau et al. 2002b). In the spinal cord of Fisher 344 rats, the age related decrease of proteasome activity was associated with both a decreased level of proteasome expression and an increased level of HNE modified β subunits (Keller et al. 2000b). In more recent studies, 26S proteasome was purified from human peripheral blood lymphocytes obtained from donors of different ages, and the patterns of proteasome subunits modified by either glycoxidation or conjugation with a lipid peroxidation product were analyzed by 2D gel electrophoresis followed by specific immunodetection of the carboxymethyl lysine or HNE adducts (Carrard et al. 2003). These modifications were analyzed, since treatment of purified proteasome with either glyoxal or HNE can inactivate the proteasome (Bulteau et al. 2001b; Conconi and Friguet 1997). Interestingly, only a restricted number of 20S proteasome subunits were modified with age, while PA700 subunits were hardly modified (Carrard et al. 2003). The question as to why some proteasome subunits are more prone to modifications than others remains to be elucidated, but the age-related increased load of modifications in certain proteasome subunits might be related to the observed inactivation of proteasome peptidase activities. Finally, the fate of the proteasome was analyzed in the human eye lens and an age-related decline in all three peptidase activities was observed (Viteri et al. 2004). This finding was consistent with a previous report from Wagner and Margolis indicating an age-related decline in proteasome peptidase activities in the bovine lens (Wagner and Margolis 1995). This decline could be explained, at least in part, by decreased proteasome content with age. However, among the three peptidase activities, the peptidylglutamyl peptide

hydrolase activity was much more decreased than the other two, indicating that this peptidase activity has been targeted for inactivation. Although this finding was only correlative and may not be related to the observed inactivation, increased glycoxidative modifications of the proteasome were evidenced with age (Viteri et al. 2004).

Proteasome activity has been reported to be inhibited by highly modified proteins such as cross-linked proteins generated upon incubation with the lipid peroxidation product HNE (Friguet et al. 1994a). Indeed, in contrast to oxidized G6PDH that becomes more sensitive to degradation, when treated with HNE, the model protein glucose-6-phosphate dehydrogenase (G6PDH) becomes less susceptible to proteolysis by the 20S proteasome. Moreover, these cross-linked proteins were found to inhibit the degradation of an oxidized protein by the proteasome in a non-competitive manner (Friguet and Szweda 1997). Thus, if present in cellular extracts of elderly individuals, such cross-linked proteins could act as inhibitors of the proteasome. Evidence for such an inhibitory mechanism has been provided since introduction of artificial lipofuscin (a ceroid pigment that accumulates in aged cells) has been shown to inhibit proteasome function (Sitte et al. 2000). More recently, accumulation of lipofuscin has been shown to result in proteasome inhibition which can induce apoptosis through the increase of proteasome regulated proapoptotic proteins (Powell et al. 2005). Conversely, proteasome inhibition can promote lipofuscin formation, suggesting that insufficient proteasomal function may contribute to lipofuscinogenesis by a compensatory increase in the amount of proteins that are directed for lysosomal degradation (Terman and Sandberg 2002). Since proteasome inhibition also induces alteration of mitochondrial homeostasis in neural cell (Hoglinger et al. 2003; Sullivan et al. 2004), the appearance of increased level of lipofuscin suggest that impairments in mitochondrial turnover may occur following proteasome inhibition. Of additional interest is the observation that proteasome peptidase activities that were strongly inhibited in rat heart homogenates from old animals, were partially restored when assayed on the purified proteasome, suggesting that endogenous inhibitors were eliminated during the purification process (Bulteau et al. 2002b). Finally, depending on the cellular system investigated, the age-related decline in proteasome activity appears to be due, at least in part, to the combined effects of: (a) decreased proteasome subunits expression; (b) direct inactivation upon modification of proteasome subunits; and (c) the presence of endogenous inhibitors such as cross-linked proteins.

4 Proteasome and Immunosenescence

Proteasomal function is generally impaired with age. Indeed, an age-related decline of proteasomal function has been documented in a variety of tissues and cell types such as rat cardiomyocytes (Bulteau et al. 2002b), human keratinocytes (Petropoulos et al. 2000), human fibroblasts (Chondrogianni et al. 2000, 2003; Merker et al. 2000) and human lens (Viteri et al. 2004). In the immune system, aging is associated with significant deficits in immune function and a decline of proteasome proteolytic

activity has been reported in lymphocytes with increasing age of human donors (Carrard et al. 2003; Ponnappan et al. 1999). It is commonly accepted that older individual fail to generate a vigorous immune response, particularly to antigens not previously encountered (Ginaldi et al. 2001; Webster 2000). This decline in immune responsiveness with age is due, at least in part, to loss of Th cell function which affect both cellular and humoral immunity (Gravekamp 2001; Weksler and Szabo 2000). Thus, the decreased B cell response to antigenic stimulation is related to Th cell deficiency and to alterations in B cell development (Kline et al. 1999). In addition to lower antigenic response, an increase in autoantigenic response is observed with advancing age (Stacy et al. 2002; Weksler and Szabo 2000). The overall decline of the immune system is linked to several pathologies such as higher susceptibility to infections, autoimmunity and cancer (Ben-Yehuda et al. 1998; Dunn and North 1991; Miller 2000). In the immune system, decreased proteasomal activity would be expected to contribute not only to accumulation of oxidized proteins but also to the lower activation of transcription factors such as NFκB, and most importantly to the lower production of antigenic peptides by the immunoproteasome for binding to MHC class I molecules.

Several studies have demonstrated the crucial role of the transcription factor NFκB in the activation of T cell through the activation of IL-2 and IL-2R genes (Pimentel-Muinos et al. 1994). The expression of the two latter have been shown to decline with age suggesting a default in their transcriptional activation. In the cytosol, NFκB is under an inactive form bound to its inhibitor IκB. The activation of NFκB occurs after stimulation by numerous agents such as cytokines (IL-1 and TNF-α), bacterial and viral infection (Ponnappan 1998). The stimulated-degradation of IκB by the proteasome declines with advancing age and results in the decreased induction of NFκB, hence contributing to the immune decline observed in the elderly (Ponnappan et al. 1999). Examination of stimulated phosphorylation and ubiquitination of IκB did not demonstrate any significant age-related alterations (Ponnappan et al. 1999). The lowered degradation of IκB was then associated to a decreased proteasome function in the elderly. Indeed, proteasome chymotrypsin-like activity was shown to decrease for T cell proteasome enriched fractions (Ponnappan 2002) and for purified 26S proteasome from human lymphocytes (Carrard et al. 2003). However, no evidence for 20S proteasome (Ponnappan et al. 1999) nor 26S proteasome (Carrard et al. 2003) decreased content was found in the elderly samples. Since the observed lower activity was not related to a decreased proteasome expression, we investigated the integrity of the proteasome structure during aging (Carrard et al. 2003). The 19S complex subunits were marginally altered upon aging since only two of its subunits, the ATPase subunits S4 and S7, were glycoxidized and/or conjugated with the lipid peroxidation product HNE. Nevertheless, it should be pointed out that S4 subunit has been implicated in 26S proteasome assembly in human cells (Mason et al. 1998). However, glycation of this subunit did not appear to affect the stability of the 26S proteasome complex, since no enhanced dissociation into 20S and 19S was observed with age. In contrast, the 20S core was much more prone to posttranslational modification during aging. Indeed, α and β subunits were overall more affected by glycation, conjugation with lipid peroxidation product

and ubiquitination in the elderly. Those modifications could have a direct impact on proteasome stability or activity. Indeed, modifications of α subunits could interfere with the accessibility of the substrate to the catalytic chamber and/or impact catalytic activities by destabilizing the interaction between regulatory α and catalytic β subunits. For example, the α7 subunit is thought to coordinate the assembly of the rest of the α subunits in human proteasome (Gerards et al. 1998) and was severely modified by glycation, conjugation with HNE and was ubiquitinated. Another interesting finding regarding lowered protease activity with age was the modification by both glycation and HNE conjugation of the iβ5 catalytic subunit which carries the chymotrypsin-like peptidase activity. Despite glycation of iβ5 in early ages, the chymotrypsin-like specific activity was not affected. However, this does not rule out the possibility that glycation occurring in samples from elderly donors may target more crucial lysine residues involved in the catalytic activity. In contrast, conjugation of iβ5 with HNE resulted in a concomitant decreased chymotrypsin-like activity of the proteasome complex. The observed increased ubiquitination of iβ5 with age may also contribute to proteasome inactivation. The specific modification of 26S proteasome subunits could be central in the defect of activation of transcriptional factors implicated in the immune response and in antigen processing. Indeed, age-related decline of proteasome in human T-lymphocytes has been recently attributed to both a lower expression of certain catalytic and structural proteasome subunits, including immunoproteasome subunits, and increased oxidative modification of proteasome subunits (Ponnappan et al. 2007). Consequently, a lower degradation of infectious protein agents by the 26S proteasome and immunoproteasome could then result directly in a higher infection level and indirectly in a lowered immune response of the elderly. An age-related up-regulation of immunoproteasome subunits has been documented in muscle and brain that could be associated with constant inflammation or oxidative stress (Diaz-Hernandez et al. 2004; Ferrington et al. 2005), while down-regulation of immunoproteasome subunits in certain tumor cells has been interpreted as an immunosurveillance escape mechanism (Kageshita et al. 1999; Meidenbauer et al. 2004; Murakami et al. 2001). Up-regulation of immunoproteasome subunits has also been documented upon treatment with oxidants arguing for the ability of the proteasome system to cope with stress and the immunoproteasome to be part of the anti-stress response (Ding et al. 2003). Interestingly, treatment of senescent fibroblasts with IFNγ, as opposed to young fibroblasts, failed to induce immunoproteasome subunits (Stratford et al. 2006). In addition, such polymorphisms of immunoproteasome subunits as the LMP2 (iβ1) codon 60 (R60H) have been associated with certain autoimmune diseases like spondylo and rheumatoid arthritis, and insulin-dependent diabetes mellitus (Deng et al. 1995; Pryhuber et al. 1996; Vargas-Alarcon et al. 2004) while an influence on susceptibility to TNFα-induced apoptosis of this particular polymorphism was observed in peripheral blood lymphocytes (Mishto et al. 2002). Therefore, investigating the age-related status of the immunoproteasome may be of critical importance due to its pivotal role in the antigen presentation pathway and both quantitative and qualitative alterations of the immunoproteasome activity would be expected to have a strong influence on the quantity and quality of immunodominant epitopes presented to T-cell receptor

of CD8+ lymphocytes, hence leading to subsequent modifications of the immune response against antigens.

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Cytokines

Age-Related Changes in Type 1 and Type 2 Cytokine Production in Humans

Elizabeth M. Gardner and Donna M. Murasko

Contents

Abstract: Aging is accompanied by several changes in immunity; however, altered T-cell function is one of the most consistent and dramatic changes observed. Because of the key roles that cyokines play in modulating the immune response, investigators have hypothesized that these age-related changes in T-cell function are related to, at least in part, by alterations in cytokine production. While data from murine studies generally support an age-related shift form a Th1-like cytokine response to a Th2-like response, data in humans do not support this age-related shift in cytokine production. This review of several studies indicates that age-associated changes in cytokine productions in humans are inconsistent. Further, these age-associated changes in cytokine production do not necessarily induce a shift to a Type 2 cytokine response. This review highlights the variables that may contribute to the inconsistent results among studies. Additional studies in humans are both

E. M. Gardner (✉) · D. Murasko
Department of Bioscience and Biotechnology
Drexel University, Philadelphia
PA 19104, USA

E. M. Gardner
Department of Food Science and Human Nutrition
Michigan State University, East Lansing, MI 48824, USA
Fax: +1517-353-8963.
E-mail: egardner@msu.edu

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critical and warranted to clearly identify the effect of altered cytokine production on age-associated changes in immune function.

Keywords: Aging • Cell-mediated response • Humans Humoral response • Immunity Type 1 cytokines • Type 2 cytokines

1 Introduction

It is well known that the incidence of cancer, infectious diseases, and autoimmune disorders increases with advancing age (Miller 1996). In addition, aging is accompanied by multiple changes in immune function, including decreased lymphoproliferative responses to both mitogens and antigens, reduced delayed type hypersensitivity reactions, and impaired antibody responses to both vaccination and infection (Miller 1996; Murasko and Gardner 2003). Thus, it has been postulated that these age-related diseases can be explained, at least in part, by an overall dysregulation in immune function (Shearer 1997).

The most consistent and dramatic age-related changes of the immune system have been demonstrated in the T-cell compartment (Miller, 1996; Murasko and Gardner 2003). Therefore, many studies have examined T-cell responses during aging to identify potential mechanism(s) responsible for these age-associated alterations in immune function. Investigators have postulated that altered cytokine production may contribute significantly to age-associated changes in immune function in both animal models and in humans. The best evidence for an age-associated dysregulation in cytokine production has been demonstrated in the mouse model. Most studies indicate that interleukin-2 (IL-2) production is consistently decreased, (Shearer 1997), while interleukin-4 (IL4) production is generally increased (Albright et al. 1995; Hobbs et al. 1993).

The above observations have led investigators to postulate that aging per se may induce a shift from a Type 1-like cytokine (IL-2, IFN-γ, IL-12) cell-mediated response to a predominantly Type 2-like cytokine (IL4, IL-5, IL-6, IL-10) response. While this hypothesis is generally supported in murine models, there is no conclusive evidence that such a shift to a dominant Th-2 response occurs in elderly humans. In fact, in 2002, we published a comprehensive review of the literature in humans to evaluate whether or not there were definitive and consistent changes in cytokine production in elderly subjects (Gardner et al. 2006). This survey of the literature did not support an age-related shift in cytokine production that favored a predominantly Th-2 response in the elderly. Since that review, little new information has emerged to support the hypothesis that there is a consistent age-related shift in cytokine production in elderly humans.

The purpose of the current review is not to reiterate our previous survey of the literature (Gardner and Murasko 2002), but rather to provide a concise summary of what is known about age-related changes in cytokine production in elderly humans, to suggest possible explanations for differences in outcomes of studies, and most important, to call for future studies to determine the impact of age-related changes on cytokine production on the immune response of the elderly.

2 Parameters that Confound Comparison of Results Among Human Studies

In our previous review (Gardner and Murasko 2002), we extensively described the various confounding variables that can influence the outcome of studies on age-related changes in human populations. These confounding variables are described briefly below and in Table 1 so that the reader is aware of possible factors that may influence the outcome of a study of immune function in elderly subjects.

2.1 Subject Populations

One of the major variables that may contribute to the outcomes of different studies is the age distribution of subjects in any given study. In order to evaluate whether or not cytokine production is altered in elderly humans, it is necessary to review the criteria utilized to select elderly subjects. First and foremost, the elderly must be at least 60 (preferably 65) years of age. It is equally important to design studies

Table 1 Parameters to consider when evaluating multiple studies on age-related changes in immune function of elderly subjects

Subject population	Advantages	Disadvantages
Age range		
Elderly: 60+ years of age Young: 18–40 years of age	Able to determine age-related changes in immune function in well-defined populations	Difficult to enroll both young and elderly subjects in the same study. Difficult to enroll elderly subjects
Health status		
Frail (nursing home) Elderly Healthy elderly Senieur (exceptional elderly)	Able to obtain information about the response of elderly populations with varying health status	Frail and Senieur elderly may not reflect age-related changes in immune function of general elderly population
Demographics		
Racial background Socioeconomic status	Similar demographics afford assessment of immune function in elderly reduces variability in subject population	Similar demographics may not reflect total elderly population, with different racial backgrounds or economic status
Sample sizes		
Large	Reduce possible skewed responses due to heterogeneity in immune response	Difficulty in enrolling a large sample number in a single study
Small	Enrollment and retention more likely	Response may be skewed and not reflect response of entire population

in which both young and elderly subjects are evaluated concurrently. Inclusion of both age groups in the same study enables investigators to clearly delineate the changes in immune function that can be attributed to old age, minimizing any variation due to assay conditions. Despite this, studies have utilized subjects who cannot be categorized as elderly (e.g., <60 years of age) and have not included a concurrent examination of young and elderly in the same study (Gardner and Murasko 2002). Therefore, the best design for assessing age-related changes in immune function, including cytokine production, is to choose subjects who meet the age requirement for elderly and to include both young and elderly individuals in the same study.

2.2 Health Status of the Subjects

A second critical component of any aging study is to clearly define the health status of the population to be evaluated in that given study. In general, human studies evaluate age-related changes in immune function in three classes of elderly: frail or nursing home elderly, exceptionally healthy (Senieur) elderly, or healthy elderly. Frail or nursing home elderly typically represents a population who is generally not in good health, having chronically debilitating disorders. Therefore, it is difficult to delineate changes in immune function that are due to age rather than to disease. However, it is still important to assess immune function in this population to identify strategies that may enhance the immune response or reduce the incidence and/or severity of infectious disease in this population. It is important to note that the changes that occur in immune function in the frail elderly may not reflect the changes seen in the healthy, free-living elderly population. The Senieur protocol was developed to limit the influence of chronic disease on age-related changes in the immune response of the elderly. The criteria employed for selection of elderly individuals in the Senieur protocol are described in detail (Ligthart et al. 1990). These Senieur elderly represents a group of individuals with exceptional health status, who are largely free from debilitating or chronic illnesses, which is not typical of the health status of the overall elderly population. Therefore, while this protocol is useful to elucidate changes in immune function that are primarily due to age and not to underlying disease, the data obtained may not be extended to the general elderly population. Finally, many studies enroll elderly who do not meet the criteria of the Senieur protocol, but whose health is generally good. In general, these individuals are independent, community-living individuals who do not have debilitating diseases, immune-related disorders or are taking chemotherapeutic agents. This population has the advantage of being more readily accessible for aging studies and results from this population are more representative of the response of the elderly population.

In summary, regardless of which population of elderly is employed in a study, it is critical to carefully define that population and to recognize the limitations of examining the chosen population for assessment of age-related changes in cytokine function.

2.3 Demographics of the Subject Population

Another consideration when evaluating age-related changes in the immune response of the elderly is the demographics of the population, which includes, but is not limited to, racial background and socioeconomic status. In order to limit variability of results, investigators typically assess age-related changes in a nearly homogeneous population of elderly. Thus, most if not all, of the subjects have the same racial (but not necessarily ethnic) background with similar socioeconomic status. While it may be argued that controlling for race and economic status can ultimately limit the variability of the results of the study, it is important to recognize that the results obtained from one subset of the elderly population may not reflect the diversity of responses within the elderly population at large. For example, most of the studies performed in the United States on age-related changes in the immune response have been generated in the Caucasian population; similiar studies in non-Caucasian elderly are seriously lacking (Marin et al. 2002; Sambamoorhi and Findley 2005).

It is recognized that subject recruitment is difficult under the best of circumstances. In fact, we recently reported strategies that we utilized to successfully enhance both recruitment and retention of elderly in human studies (Gonzales et al. 2007). The lack of data in non-Caucasian groups may not be intentional, but rather, may reflect the inability of investigators to gain access to these populations for their studies. Secondly, the under-representation of non-Caucasians may also reflect a lack of trust of elderly subjects at the time of recruitment (Gonzales et al. 2007). Our laboratory recently conducted a study to evaluate age-related changes in the immune response of a racially-diverse elderly population. The results of this study (Gardner et al. 2006), which will be discussed below, clearly indicate that immune response data generated from Caucasian elderly do not necessarily reflect the responses of subsets of non-Caucasian elderly. Therefore, additional studies in racially diverse populations are warranted to provide conclusive data regarding age-related changes in the elderly population at large.

2.4 Sample Size of the Population

We (Murasko et al. 1991) and others (Barcellini et al. 1988) have documented that the immune response of elderly subjects shows marked heterogeneity. It has been postulated that several factors may contribute to this heterogeneity and include factors such as health status, genetic variability, and behaviors, such as diet, smoking, level of physical activity, or cognitive status (Ritz and Gardner 2006). Although many studies have consistently shown that the mean proliferative responses to either nonspecific stimulation or antigenic stimulation are reduced in the elderly compared to young individuals (Bernstein et al. 1998; Gardner et al 2006); we have clearly shown that there are some elderly who produce responses that are nearly equivalent to those of young, while others produce responses that are only about

20% of the response of the young (Murasko et al. 1997). Therefore, it is necessary to employ large samples to control for this heterogeneity in the immune response of the elderly. However, many studies in the literature have assessed immune function on a small number of elderly. Therefore, it is plausible that small sample sizes may unintentionally select for responses that are either very high or very low. Thus, the data generated on a small cohort of elderly subjects may not be indicative of a larger elderly population and can contribute to differences in the outcomes of studies on age-related changes in the immune response even when other variables, such as health status, are controlled. Investigators should perform a statistical power analysis to determine the number of subjects needed to offset the expected heterogeneity of the immune response in an elderly population.

3 Age-Related Changes in Cytokine Production

Evaluation of age-related changes in cytokine production has been the focus of many studies in which both young and elderly individuals have been assessed concurrently. It would seem, therefore, that a comprehensive review of these reports should definitively answer the question of whether or not there is an age-associated dysregulation in cytokine production in elderly humans. Unfortunately, this has not been the case; differences in experimental conditions, such as the stimulating agent, the tissue employed, and the time points of evaluation, make it difficult to compare the results of all studies and to draw general conclusions from them.

Typically, cytokine production has been assessed in peripheral blood mononuclear cells (PBMC), but some investigations have utilized cultures of whole blood or have isolated specific subsets of lymphocytes for analysis. Likewise, some studies have evaluated cytokine production in response to a nonspecific stimulus, such as PHA, while others after specific stimulation, such as influenza. Clearly, differences in outcomes may reflect the tissue analyzed as well as the stimulating agent utilized to induce cytokine production. Both the time points and the methods of assessments vary considerably among studies. In most cases, cytokine production is measured at only one time point (e.g., 48 or 72 hrs after stimulation); therefore, it is possible that differences in outcomes of reports may simply reflect kinetic differences in the peak of the response to a particular cytokine. Finally, comparison of reports on age-related alterations in cytokine production is made more difficult by the various techniques used to quantitate cytokines. Some studies have measured cytokines in supernatants from stimulated cells by bioassays, while others have employed enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays (RIA). Bioassays assess the functional activity of a sample using either growth or inhibition of growth of cell lines specifically responsive to that particular cytokine (e.g., IL-2, IL-4, IL-6) or inhibition of viral cytopathic effect (i.e., IFN). In contrast, both ELISA and RIA measure the total amount of a cytokine by using antibodies specific for antigenic determinants of the cytokine.

While ELISA and RIA are highly sensitive and specific in quantitating cytokine concentrations, they do not provide any information about the functional activity of the cytokines measured. Importantly, it is difficult to compare the results of studies on the same cytokine when one employed a bioassay and the other used ELISA since these results do not necessarily correlate (Murasko, unpublished data).

Some studies have evaluated cytokine mRNA produced by cells of young and elderly to avoid the problems of measuring proteins in the supernatants of stimulated cells. While the levels of mRNA provide useful information regarding age-associated alterations in transcription of cytokine genes, it is important to recognize that mRNA results do not always reflect the amount of protein produced and secreted. For example, we have reported previously (Gardner and Murasko 2002), elderly individuals who had increased levels of IFN-γ mRNA had comparable levels of biologically active IFN-γ relative to young controls.

It is, therefore, difficult to resolve disparities among studies when different methods of cytokine analyses have been employed. Clearly, the combination of these variations in experimental design could significantly contribute to the varying outcomes, even when all other parameters of the study population are controlled. It is quite possible that differences among studies of humans may reflect even subtle differences in experimental design.

In summary, appropriate evaluation of the current literature of changes in cytokine production with age requires careful consideration of the health of the subjects, the demographics of the population, the cell types and stimuli used, and the techniques employed to measure cytokines. Additional comprehensive evaluations in studies that utilize appropriate and well-controlled experimental designs are absolutely critical to identify the impact of altered cytokine production on immune function in the elderly.

4 Age-Related Changes in Type 1 and Type 2 Cytokines

In human studies, IL-2 and IFN-γ are the most frequently measured cytokines for characterization of Type 1 cytokine responses, while ILs-4, 6, and 10 are often employed to characterize Type 2 cytokine responses. In order to make general conclusions regarding the effect of cytokine dysregulation on age-associated changes in immune function, the data from several reports in which Type 1 or Type 2 cytokines were assessed from either healthy or Senieur elderly, along with young subjects in the same study, are summarized below. For simplicity and ease of comparison among studies, this review will mainly focus on studies in which cytokine production was assessed in PBMC after mitogenic or antigenic stimulation. However, when appropriate, a discussion of those studies in which cytokine production was assessed in isolated immune cells, whole blood, plasma, or sera will be included. These criteria regarding the studies reviewed in this chapter were selected in order to draw broad conclusions regarding cytokine dysregulation in the elderly.

5 Type 1 Cytokines

5.1 Interleukin-2

One of the most consistent age-related changes in immune function is decreased T-cell lymphoproliferative responses (Miller 1996; Murasko et al. 1987). Since interleukin-2 (IL-2) is necessary for the activation and proliferation of T lymphocytes (Janeway et al. 2005), it stands to reason that age-related changes in IL-2 production have been assessed in several studies in the elderly. Age-related changes in IL-2 production have been measured in elderly subjects under various culture conditions and in response to nonspecific stimulation or after stimulation with specific antigens, such as influenza.

The overall results of several studies in which IL-2 production was evaluated are summarized in Table 2. In a survey of fourteen studies, in which PBMC or whole blood from young and elderly subjects were stimulated with PHA, an age-related decrease was observed in eleven reports (Barcellini et al. 1988; Born et al. 1995; Gardner et al. 2000, 2006; Gillis et al. 1981, Murasko et al. 1991; Nagel et al. 1986; Orson et al. 1989; Song et al. 1993; Wu et al. 1994; Xu et al. 1993), while there was no age-related difference in three studies (Bruunsgaard et al. 2000; Sindermann et al. 1995; Weifang et al. 1996). However, the two studies (Bruunsgaard et al. 2000; Sindermann et al. 1995) reporting no change in IL-2 levels stimulated whole blood with PHA, rather than PBMC. Interestingly, in one of the more recent studies (Gardner et al. 2006), PHA-induced IL-2 production was assessed in PBMCs from a racially-diverse group of elderly, consisting of 33 Caucasians, 39 African Americans and 41 Latinos. This study demonstrated that IL-2 production was reduced in all elderly, regardless of racial background, relative to young controls assessed con-

Table 2 Summary of age-related changes in type 1 cytokines[a]

Cytokine	Assessment	Stimulus	Age-related Changes[b]
IL-2	PBMC	PHA	Decreased (11 reports) No change (1 reports)
	Whole Blood	PHA	No change (2 reports)
	PBMC	Trivalent influenza vaccine	Decrease (5 reports) No change (1 report Latino)
	PBMC	Live influenza virus	Decrease (2 reports) No change (2 reports)
IFN-γ	PBMC	PHA or ConA	Decreased (4 reports) No change (2 reports) Increased (1 report)
	Whole blood	PHA	No Change (2 reports)
	PBMC	Influenza vaccine	Decreased (4 reports) No change (1 reports)

[a] Adapted from (Gardner and Murasko 2006)
[b] Results are compared to young controls

Table 3 Summary of age-related changes in Type 2 Cytokines[a]

Cytokine	Assessment	Stimulus	Age-related changes[b]
IL-4	PBMC	PHA, ConA	No change (2 reports)
		Anti-CD2/Anti-CD28	Increased (1 report)
		Anti-CD3/PMA	Decreased (1 report)
	PBMC	Influenza vaccine	Undetectable (2 reports)
IL-6	PBMC	PHA or ConA	No change(3 reports)
			Increased (2 reports)
			Decreased (report)
	Serum	None	No change (3 reports)
	Plasma		Increased (2 reports)
IL-10	Blood	PHA	Decreased (1 report)
	PBMC	Influenza vaccine	Increase (1 report)
			Decreased (2 reports)
	PBMC	None	No change (2 reports)
	Serum	None	No change (1 report)

[a] Adapted from (Gardner and Murasko 2006)
[b] Results are compared to young controls

currently. This observation of decreased IL-2 production in non-Caucasian elderly has been confirmed in a subsequent study in our laboratory (Gardner and Murasko, unpublished data). Therefore, the majority, but not all, of studies demonstrate an age-related decrease in PHA-induced IL-2 production, relative to young controls.

Several studies (Gardner et al. 2006; McElhaney et al. 1990, 1992, 1995; Quyang et al. 2000) have also evaluated IL-2 production in the elderly after stimulation with influenza to determine the response to a specific antigen. In two reports (McElhaney et al. 1990, Quyang et al. 2000), when PBMC were stimulated with trivalent influenza vaccine, the elderly produced significantly less IL-2 after vaccination than did young controls. Interestingly, a more recent influenza study (Gardner et al. 2006) in a racially-diverse elderly population demonstrated that PBMC from elderly Caucasians and African Americans, but not from Latinos, produced significantly less IL-2 after influenza vaccination, relative to that produced by young controls. When various strains of live influenza virus were employed, mixed results were obtained. Studies have indicated that IL-2 production after stimulation of PBMCs with influenza virus was either reduced or unchanged (McElhaney et al. 1992, 1995), depending on the strain of influenza utilized, relative to the response of young controls.

Therefore, a careful review of current literature clearly suggests that while many elderly produce less IL-2 than young, not all elderly demonstrate an age-related decrease in IL-2 production. Importantly, recent data also suggests that racial background must be considered when evaluating age-related changes in IL-2 production. The reasons for these disparate results among studies are not clear. However, possible reasons include differences in sample numbers assessed within various studies, the overall heterogeneity in the immune response of the elderly, and the type of stimulus. Based upon this review, decreased in IL-2 production cannot be presented as a definitive age-associated alteration in humans.

6 Interferon-γ (IFN-γ)

IFN-γ is secreted mainly by T lymphocytes and NK cells and is known for inducing antiviral activity, upregulating MHC class I and II antigens, and activating macrophages (Janeway et al. 2005). Since IFN-γ is a strong inducer of cell-mediated immune (CMI) responses, investigators have hypothesized that age-related changes in IFN-γ may play a role in the decline of CMI with age (Shearer 1997).

There have been several reports that have assessed the effect of age on IFN-γ production in the elderly. The results of these studies are summarized in Table 2. When changes in IFN-γ production by PBMC after stimulation with either PHA or ConA were evaluated in young and elderly subjects, four studies reported an age-associated decrease (Born et al. 1995; Candore et al. 1993; Lio et al. 1998, 2000), two studies demonstrated no change (Hessen et al. 1991; Weifeng et al. 1986), and one showed an increase in IFN-γ (Murasko et al. 2001), relative to the response of young subjects. Two other studies in which cultures of blood were stimulated with PHA reported no differences in IFN-γ production between young and elderly individuals (Bruunsgaard et al. 2000; Sindermann et al. 1993). Although the exact reasons for the differences in outcomes among these reports are not clear, a review of these studies clearly indicates that the experimental designs among studies varied considerably. There were differences in length of stimulation in vitro (1–5 days) and in the techniques used to measure IFN-γ (biossay versus ELISA). Therefore, the discrepancy among studies may simply reflect altered kinetics of IFN-γ production or variations in assays used for quantitation of IFN-γ.

Studies in which PBMC were incubated with specific stimuli using influenza antigen generally support an age-related decrease in IFN-γ. Five studies demonstrated decreased IFN-γ production to influenza virus in the elderly, relative to young controls (Bernstein et al. 1998, Gardner et al. 2006, McElhaney et al. 2006; Murasko et al. 2001; Quyang et al. 2000;). In one study (Bodnar et al. 1997), IFN-γ production by PBMC from elderly after stimulation with influenza showed a non-significant decrease relative to that produced by young subjects. This lack of statistical age-related decrease in the elderly was likely due to the small number of subjects included in the study. Our recent study (Gardner et al. 2006) in a racially-diverse elderly population also supports an age-related decrease in IFN-γ production, with both the total elderly, as well as all elderly subgroups, producing less IFN-γ after influenza vaccination compared young controls. However, an interesting observation that emerged from this study was that IFN-γ levels decreased from pre- to post-vaccination in elderly African Americans, but not in any of the other groups of elderly individuals or in the total elderly population. This observation was confirmed in a subsequent study (unpublished observations). These data suggest that racial background can influence age-related changes in the cell-mediated response. Importantly, these altered responses of elderly African Americans, relative to the total elderly population, may not have emerged had the elderly not been categorized by racial background. Future studies are necessary to validate these findings.

In a recent report, McElhaney et al. (2006) questioned whether or not indices of CMI could be utilized to distinguish between elderly individuals who did or did not develop laboratory diagnosed influenza (LDI). In this study, 90 elderly (60 years and older) and 10 healthy young adult controls were immunized with the 2003–2004 trivalent inactivated influenza vaccine. The study reported that 9 out of 90 elderly developed LDI during the course of the study. Before vaccination, subjects who developed LDI had 10-fold lower levels of IFN-γ after stimulation with live influenza virus compared to those elderly who did not develop LDI. Although the subjects without LDI showed no significant change in IFN-γ levels over the course of the study, the older adults who developed LDI showed significant increases in IFN- γ levels in influenza-stimulated PBMCs. The mean IFN-γ:IL-10 ratio in influenza-stimulated PBMC was 10-fold lower in LDI versus nonLDI subjects. These results are important because they correlate cytokine production with LDI in the elderly and also argue for altered Type 1 and Type 2 cytokine responses in the elderly. Clearly, future studies should confirm this observation in a larger population of elderly with or without LDI.

In summary, a careful review of the current reports suggest that there are no consistent age-related changes in IFN-γ production after non-specific stimulation, at least when PHA or ConA are utilized. Until additional studies that carefully compared dose and kinetics are performed, no definitive conclusions can be made. In contrast, the data to date are fairly consistent in demonstrating an age-associated decrease in IFN-γ production upon antigen-specific stimulation, at least when influenza is the antigen used.

7 Type 2 cytokines

7.1 Interleukin 4

IL-4 is a Type 2 cytokine secreted by T-cells, B cells, macrophages, mast cells, and basophils and induces B cell differentiation and antibody class switching (Janeway et al. 2005). It has been demonstrated that IL-4 plays a critical regulatory role in inhibiting the production of Type 1 cytokines, while stimulating the production of Type 2 cytokines (Shearer 1997).

Assessment of age-related changes in IL-4 production in humans in response to mitogenic stimulation is quite limited and the results of these studies are not consistent (Gardner and Murasko 2002). While two reports showed that stimulation with either PHA (Candore et al. 1993) or ConA (Bernstein et al. 1998) resulted in comparable IL-4 production by both young and elderly, there are reports to indicate either an age-related increase (Nijhuis et al. 1994); or decrease (Karanfilov et al. 1999) in IL-4 production. However, those studies indicating an age-related increase, measured IL-4 production by PBMCs after stimulation with a combination of anti-CD2/anti-CD28, whereas the report demonstrating an age-related increase utilized a

combination of CD3 and PMA. Therefore, differences in IL-4 production after nonspecific stimulation may be dependent on the agent used to induce the response.

Attempts have been made to evaluate the age-related changes in IL-4 production by PBMC after stimulation with specific antigens. When PBMCs from young or elderly subjects were stimulated with either trivalent whole inactivated influenza vaccine (Bodnar et al. 1997) or after stimulation of PBMC with trivalent influenza subvirion vaccine (Bernstein et al. 1998), IL-4 levels could not be detected. It is possible that increasing age has either no effect on IL-4 production or that the effect is not very robust, since variations in experimental design result in very different outcomes. Additional studies that address these experimental issues are necessary before the effect of age on IL-4 production can be ascertained.

7.2 Interleukin 6

IL-6 is a Type 2 cytokine that impacts both T and B cell responses, and is a major component of the acute phase inflammatory response. The major cells types that produce IL-6 include T-cells, monocytes, macrophages and mast cells (Janeway 2006). T-cell activation and differentiation, B cell differentiation and mucosal IgA responses are all induced by the production of IL-6.

IL-6 has been deemed a "gerontologist cytokine" because it has been postulated that advancing age is associated with increased IL-6 levels (Ershler et al. 1993). However, a careful review of the literature to date does not support this claim, at least in human studies. Several studies have assessed the impact of age-related changes on IL-6 production after stimulation of PBMC or whole blood with mitogens. Stimulation with PHA (Candore et al. 1993; Beharka et al. 2001) or a combination of PHA and PMA (Fagiolo et al. 1993) induced comparable IL-6 production from PBMC of elderly and young after 24 hrs of incubation. However, a longer stimulation with PHA and PMA induced higher levels of IL-6 in elderly at 48 and 72 hrs of stimulation, while IL-6 levels remained constant in the young from 24–72 hrs (Fagiolo et al. 1993). It is not certain if this age-related increase is due to the type of stimulus (e.g., the addition of PMA to the culture) or reflects actual kinetic differences between young and elderly. Clearly, measurement at later time points may indicate an age-related increase in IL-6. However, an additional study argues against age-related kinetic differences since whole blood incubated with PHA for 96 hrs induced comparable levels of IL-6 in young and elderly subjects. The possibility that the inducing agent influences IL-6 production can not be excluded. A well-defined study by Beharka and colleagues measured IL-6 production by PBMCs from the same individuals after stimulation with PHA or ConA in fetal bovine serum (FBS) or autologous plasma (AP). While PHA in AP, PHA in FBS, and ConA in FBS induced comparable levels of IL-6 in young and elderly, IL-6 was decreased when ConA in AP was utilized, relative to young controls. Collectively, it appears that kinetic differences as well as the stimulating agents influence IL-6 production after nonspecific stimulation. Future studies in which a detailed kinetic

analysis is performed using the same stimulating agent are required to definitively determine the effects of age on IL-6 production.

The effect of age on IL-6 production by PBMC stimulated with specific antigen has only been evaluated in response to influenza vaccine (Bernstein et al. 1998). In this study, IL-6 production was comparable between young and elderly subjects when PBMCs were stimulated in vitro with trivalent influenza vaccine before and after influenza immunization. However, it is important to note that there was considerable heterogeneity in IL-6 responses in both young and elderly before and after influenza vaccination. Therefore, if small numbers of subjects are evaluated, it is possible that a higher IL6 response in the elderly may reflect a sampling error rather than true biologic differences.

It has been suggested that concentrations of IL-6 in plasma or serum increase with advancing age (Ershler et al. 1993; Kania et al. 1995). Although the investigators who support this hypothesis recognize that IL-6 is usually undetectable in the absence of inflammation, (Ershler 1993), they still believe that this increase is solely due to age and not symptomatic inflammation. However, this conclusion is difficult to support since the elderly subjects in these reports, although defined as healthy elderly, were not screened for inflammatory diseases, such as arthritis. We have found in a previous influenza study that IL-6 levels were significantly elevated in a subset of elderly individuals prior to vaccination with influenza (Bernstein et al. 1998). These individuals did not produce a significant increase in IL-6 after vaccination with influenza. When the health status of these individuals was analyzed retrospectively, those individuals with increased IL-6 levels had reported that they did have arthritic flare-ups (Bernstein et al. 1996). Three additional studies evaluating IL6 levels in plasma and sera have observed no age-associated difference (Beharka et al. 2001; James et al. 1997; Peterson et al. 1994). In one study (Beharka et al. 2001) it was reported that there was no age-associated increase in IL-6 among the elderly; interestingly, subjects that were in the 65–69 and 75–80 age groups had higher IL-6 levels than those in the 70–74 and > 80 age groups. Collectively, these studies suggest that both health status and genetic heterogeneity may be a major factor in the variation in IL6 production observed among studies.

Overall in the human system, the data for an age-related increase in IL-6 is not convincing. Studies using similar techniques and subject populations have reported contrasting results. While there is strong evidence for elevated levels of IL-6 in disease states (Ershler, 1993) that are associated with aging, in the absence of disease, the current data does not support an age-associated change in IL-6 production.

7.3 Interleukin-10

IL-10 is produced by T and B cells, monocytes, and macrophages and inhibits macrophage activity by inhibiting cytokine production and downregulating MHC class II antigen expression (Janeway 2006). Like IL-4, IL-10 plays a key

regulatory role in inhibiting production of Type 1 cytokines (Shearer 1997), thus down-regulating CMI responses. Investigators have hypothesized that an age-related increase in IL-10 production may influence the age-related decrease in CMI. Recent data from McElhaney et al. (2006) support this hypothesis since IL-10 levels increased after ex vivo stimulation of PBMC with influenza following immunization of elderly subjects, regardless of whether or not they had LDI. However, LDI subjects had threefold higher levels of IL-10 production by PBMC after ex vivo stimulation with influenza, compared with non-LDI subjects. This suggests that those elderly who develop LDI may favor a more Th-2 like response due to altered IFN-γ:IL-10 ratios.

Earlier studies, however, have utilized a number of culture conditions to evaluate production of IL-10 by PBMC or whole blood. Basal IL-10 production by PBMC cultured for 24 hrs without stimulation showed comparable levels in young and elderly (Llorente et al. 1997). The only study examining IL-10 in serum found comparable levels in young and elderly subjects (Peterson et al. 1994). Stimulation of PBMCs with trivalent influenza vaccine prior to immunization (Bernstein et al. 1998) also demonstrated no age-related differences. However, production of IL-10 after stimulation of PBMC with trivalent influenza vaccine or influenza B after immunization resulted in significantly decreased IL-10 production in the elderly compared to young (Bernstein et al. 1998; Llorente et al. 1999). A similar age-associated decrease was observed after stimulation of whole blood with PHA for 24 hrs (Bruunsgaard et al. 2000).

Therefore, similar to the data with other cytokines, conflicting results have been observed with IL-10. The data on IL-10 production range from being decreased, unchanged or increased, and are influenced by the stimulus or tissue examined. The age-related changes in influenza vaccine-induced IL-10 production that is observed only after influenza vaccination indicate that altered IL-10 production in response to specific stimuli is subtle and may be unmasked only by in vivo immune challenges, such as illness or vaccination. However, due to the differences in stimuli and the limited number of studies, a definitive conclusion is not possible at this time.

8 Conclusions and Future Directions

It is well established that immune function declines with advancing age in both humans and in animal models. Since cytokines are key components in the regulatory communication that occurs among immune cell, it is likely that altered cytokine production may contribute to these age-associated changes in immune function. Murine models of aging have shown an age-regulated dysfunction in cytokine production, as evidenced by consistently decreased IL-2 and increased IL-4. These data, coupled with the increased incidence of cancer and virus infections in the elderly have led investigators to hypothesize that aging may favor a predominant Type 2 cytokine response. Therefore, the purpose of this chapter was to evaluate the current literature regarding age-related changes in cytokine production in the elderly to determine whether the preponderance of evidence supports this contention.

Our current review of the literature cannot support the contention that there is, in fact, an age-associated shift to a predominant Type 2 cytokine response in the elderly. Despite the large number of studies that have been conducted over the last several years, differences in experimental design make it extremely difficult to compare the data among studies. This review clearly shows that factors such as age, health status, genetic heterogeneity and the demographics of the elderly population, all influence the outcome of a study. Likewise, differences in stimulating agent, its dose, time points of assessments and method of assessment for cytokine production all greatly influence the results of human studies. Therefore, without some way to control for the confounding variations in experimental design, a definitive conclusion among studies cannot be made. In epidemiologic studies, a meta analysis of raw data from several studies is often employed to draw conclusions regarding a biologic outcome from studies that do not have the same design. Perhaps a meta analysis of the studies evaluating cytokine production in the elderly may reveal associations that have not been apparent by simply reviewing the published data.

In order to validate the contention of an age-related shift in cytokine production, it is necessary to assess both Type 1 and Type 2 cytokines concurrently in the same study. While this concurrent analysis has been done in some of the studies reviewed, it has not been reported in all of them. Likewise, it is not acceptable to measure only one cytokine falling into either category and suggest that a predominant Type 1 or 2 response has been achieved. Rather, it is necessary to measure a panel of cytokines that may be induced during the response being assessed. Such an analysis can easily be performed with the development of multiplex cytokine bead arrays, in which several cytokines can be assessed from the same sample.

In summary, based on the current data, the reader is left with more questions than answers regarding the effect of cytokine dysregulation on age-related changes in immune function. It is clear that there are age-related changes between young and elderly in cytokine production; however, more comprehensive studies are required to assess the influence of age-associated cytokine dysregulation on the immune response of the elderly. Further, while assessment in response to mitogens may reflect the potential of cells, it is the response to natural, environmental stimuli, such as infectious agents that are most important. Therefore, is important for these studies to focus on the immune response to infectious agents, rather than to nonspecific stimulation, so that effective cytokine treatments may be developed to enhance vaccination strategies and immunotherapeutic targets.

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Cytokine Expression and Production Changes in Very Old Age

Susan E. McNerlan, Marilyn Armstrong, Owen A. Ross and I. Maeve Rea

Contents

1 Introduction

Ageing is associated with various changes in immune parameters, alterations in lymphocyte subsets and cytokine dysregulation (Cossarizza et al. 1997). Cytokines are central to the regulation of the immune-inflammatory response in old age and so perhaps play a pivitol role in ageing and survival. But whether these alterations in cytokine expression and production are the secret of long life or are an indication of underlying disease, even in the apparently healthy, is uncertain. While studies of cytokine gene polymorphisms suggest that certain cytokine genotypes are associated with long life (Rea et al. 2006), cytokine levels have also been associated with various age-related diseases (Forsey et al. 2003). Studies of these parameters in very

I. M. Rea (✉) · S. E. McNerlan
Department of Geriatric Medicine
School of Medicine
Dentistry and Biomedical Sciences
Queen's University, Belfast
Whitla Medical Building
97 Lisburn Road
Belfast BT7 9AB, UK
E-mail: i.rea@qub.ac.uk; s.mcnerlan@qub.ac.uk

M. Armstrong
School of Medicine
Dentistry and Biomedical Sciences
Queen's University, Belfast

O. A. Ross
Mayo Clinic
Jacksonville, USA

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elderly subjects, i.e., those who have aged successfully, are perhaps the most useful in determining the key to longevity.

Cytokines have been investigated extensively in elderly people, at times with conflicting results. This is possibly in part due to the number of different methodologies employed. Immunoassays have been used for the measurement of circulating cytokines in plasma. However, due to the detection limits of kits, and the presence of natural inhibitors, soluble receptors or antagonists, their presence in serum may be masked. Bioassays, involving in vitro stimulation of whole blood or separated mononuclear cells, have also been widely employed in the study of age-associated changes in cytokine production. However, the response of cells to stimulants in an unnatural environment may not reflect what occurs in vivo. Also neither bioassays or immunoassays give an indication of the exact cellular source of these growth factors. Intracellular cytokine detection is a relatively new methodology which enables detection of cytokines at a single cell level thereby identifying the specific cell subsets producing these mediators (Jason et al. 1997; Jung et al. 1993; Prussin et al. 1995). The technique is performed in whole blood so cells can be kept in their natural environment. Still, while each of these methodologies have their limitations, a great deal of information on the cytokine profile of very elderly subjects has still been and is continuing to be elucidated.

2 IL-6

Interleukin 6 (IL-6) has been described as a "cytokine for gerontologists" (Ershler, 1993). It plays a key role in the acute phase response and displays both proinflammatory and anti-inflammatory activities. It is normally present in low levels in the blood, with increased levels detected during infection or trauma. Interleukin 6 levels have been widely reported to be elevated in the serum of very elderly subjects (Cohen et al. 1997; Forsey et al. 2003; Giuliani et al. 2001; Wei et al. 1992). Giuliani et al, in a study of 220 women aged 25–104 years, including 22 centenarians, showed that serum IL-6 levels increased exponentially with age. In the same group, soluble IL-6 receptor, which enhances IL-6 activity, and soluble gp130, an IL-6 inhibitor, increased until the 7th decade of life before decreasing in the older age groups.

The mechanisms behind this increase in IL-6 levels with age, as well as the cellular sources are still not fully understood. IL-6 production by both stimulated and unstimulated leucocytes is increased in elderly subjects by both PBMN cells and monocytes (Rea et al. 1995; Rink et al. 1998; Roubenoff et al. 1998). Using the technique of intracellular cytokine detection by flow cytometry, O'Mahoney et al. (1998) showed a statistically significant increase in intracellular IL-6 production in CD3+ T-cells and an insignificant increase in monocytes.

A number of studies have linked IL-6 polymorphisms to longevity, however, results have been conflicting. In a study of Italian centenarians, the 174G/C promoter polymorphism in the IL-6 gene, the GG genotype was decreased in male

centenarians and was associated with increased plasma IL-6 levels. This would suggest that those genotypes producing high levels of IL-6 appear to be detrimental to long life, at least in men; there was no difference detected in women (Bonafe et al. 2001). Studies of Irish octo- and nonagenarians have also shown the GG genotype to be decreased in the elderly (Rea et al. 2003; Ross et al. 2003a). Other studies, however, have shown no difference in genotype frequencies between centenarians and young controls (Carpurso et al. 2004; Pes et al. 2004). The reasons for these discrepancies are unclear, however, cultural and lifestyle differences between the populations studied may play a role.

This increase in IL-6 levels, rather than being the key to longevity, may instead be a reflection of an increased inflammatory state caused by underlying disease even in the apparently well elderly person. Elevated IL-6 levels have been reported to be associated with several age-related diseases including coronary heart disease, arthritis and osteoporosis (Forsey et al. 2003). High levels are also associated with a decline in function and cognitive ability and also in stroke (Barbieri et al. 2003; Cesari et al. 2004; Cohen et al. 1997; Ershler and Keller 2000). It has also been indicated as a strong predictor of mortality in elderly people (Bruunsgaard et al. 2003a; Harris et al. 1999; Volpato et al. 2001) but not in persons aged 100 years (Bruunsgaard et al. 2003b).

3 TNF-α

TNF-α is another pro-inflammatory cytokine and an important mediator of the immune response. It is widely reported as being elevated in the plasma of elderly people and levels have been found to correlate with IL-6, sTNFR and CRP in centenarians (Rea et al. 1999; Bruunsgaard et al. 1999, 2000, Sandmand et al. 2003). Increased production from unstimulated monocyte monlayers has also been reported (McNerlan et al. 1997), However, LPS stimulated leucocytes have yielded conflicting results (Bruunsgaard et al. 2003a).

Using intracellular cytokine detection by flow cytometry, the percentage and absolute counts of CD3+ T-cells producing TNF-α were significantly higher in a study of very healthy octo- and nona-genarians compared to young controls (McNerlan et al. 2002). In another study of slightly younger individuals, >62 years (mean age 73), there were significant increases in intracellular T-cell TNFα and an insignificant increase in monocyte TNF-α (O'Mahoney et al. 1998). However, a Danish study which showed increased circulating TNF-α with increasing age in a cohort including centenarians only found an increase in the percentage and number of T-cells expressing TNF-α in the group of 81 year olds but not in the centenarians, suggesting that T-cells contribute to the increased TNF-α levels in elderly subjects but other mechanisms must come into play in the much older individual (Sandmand et al. 2003).

Polymorphisms of the TNF-α gene do not appear to be associated with longevity (Rea et al. 2006). Three studies of Finnish nonagenarians, Italian centenarians and

Irish nonagenarians showed no difference in the frequency of the TNFα-308A/G polymorphism compared to young controls (Lio et al. 2003; Ross et al. 2003b; Wang et al. 2001). No significant sex differences emerged either. There is, however, a reported association with Alzheimers disease. A haplotype for TNFα associates in siblings with late onset AD and carriers of -308A show an earlier mean age at onset (Alvarez et al. 2002; Collins et al. 2000; McCusker et al. 2001). High plasma levels of TNF-α were found to be associated with moderate to severe dementia in a cohort of Danish centenarians (Bruunsgaard et al. 1999), however, it is unclear whether its role is causative or if it is the result of an increased immune activation caused by the underlying pathologic processes.

TNFα is evident in other disease processes associated with ageing. High levels of TNF-α were seen in a study of 130 octogenarians with atherosclerotic CVD (Bruunsgaard et al. 2000), and in a group of centenarians with generalized atherosclerosis (Bruunsgaard et al. 1999). Higher levels of TNF-α were found in a study of 70-year-old men with type II diabetes mellitus compared to age-matched controls and levels were found to correlate with the severity of insulin resistance (Nilsson et al. 1998). High levels of both TNF-α and IL-6 were associated with lower muscle mass and muscle strength in older men and women (Visser et al. 2002).

In a study of 333 relatively healthy 80 year olds, TNF-α was found to be associated with mortality in men but not women (Bruunsgaard et al. 2003a), whereas in a group of centenarians, recruited around their 100th birthday, elevated TNF-α was associated with mortality in both men and women (Bruunsgaard et al. 2003b).

4 Other Pro-Inflammatory Cytokines

Ageing is characterized by a low grade increase in inflammatory markers. In addition to IL-6 and TNFα, another primary mediator of the inflammatory response is IL-1. Reports on the production of IL-1β from cells from elderly people have been conflicting with reports of increased, decreased and no difference (Krabbe et al. 2004). Differing results may be due to different cell populations (WB, PBMC, monocytes,etc.) and the stimulants used (LPS, PMA, etc.). The InCHIANTI Study of subjects >65 years of age found no relationship between serum levels of IL-1β and age but found levels were associated with heart failure and angina (Di Iorio et al. 2003).

IL-18 is another proinflammatory cytokine associated with various major disabling conditions, including ischemic disease. However, whether it is the cause or a byproduct of these events is uncertain. Serum IL-18 levels are higher in centenarians compared to a young control group and also compared to a group of patients with chronic ischemic syndromes (Gangemi et al. 2003). These authors also report a significant increase in circulating levels of IL-18 binding protein, a natural inhibitor, compared to the other 2 groups which would explain the apparent paradox of elevated IL-18 with no vascular disease in these centenarians. Another study of 1671 elderly subjects aged 65-80 years showed elevated IL-18 levels to be associ-

ated with a decline in physical function, and that a polymorphism in the IL-18 gene which reduces IL-18 serum concentration, was associated with improved walking speed (Frayling et al. 2007).

IL-8 is a neutrophil chemotactic factor and inflammatory cytokine which brings neutrophils to the site of inflammation to contain infection (Baggiolini, et al. 1992). Increased levels have been detected after LPS stimulation of leucocytes from elderly individuals (Rink et al. 1998). IL-8 has been proposed as a possible key to longevity in a small study of centenarians. A study of 30 young people (21–37 years), 30 healthy elderly (65–87 years) and 10 centenarians found levels of IL-8 to be elevated in the serum of the centenarians compared to the other two groups (Wieczorowska-Tobis et al. 2006), while IL-6 levels were unchanged. This might suggest that increased serum IL-8 alongside low IL-6 might be related to longevity, although larger studies are needed to confirm this finding. However, Ross et al. (2003a) found that while AA homozygotes of the IL-8 -251 A/T polymorphism are associated with higher production levels of IL-8, there was no significant difference in IL-8 -251 A/T polymorphisms in a group of nonagenarians compared to young controls, but the study was relatively small.

IL-12 is a central cytokine acting on T- and NK cells directing proliferation of activated T-cells towards a Th1 phenotype (Trinchieri 1993). It is an important cytokine in the early inflammatory response where it stimulates IFNγ production from T- and NK cells. It is a disulphide linked heterodimer composed of a p40 heavy chain and a p35 light chain. The heterodimer IL-12p70 equates with biological activity whereas the homodimer IL-12p40 acts as an IL-12 antagonist in vitro (Mattner et al. 1993). Several studies have investigated age-related IL-12 production by mitogen-stimulated PBMCs in elderly people but results have been conflicting (Tortorella et al. 2002). In a study of very elderly subjects there was no difference in the IL-12A/C polymorphisms with ageing (Ross et al. 2003a).

However, in a study of very elderly subjects (Irish octo/nonagenarians), serum levels of total IL-12, IL-12p40 and the IL12p40/IL-12 p70 ratio, but not IL-12p70, were increased significantly with age (Rea et al. 2000). This increase in total IL-12 and the p40 subunit may be part of the cytokine dysregulation evident in the elderly or there may be an age-related imbalance in the transcription of the p40 and p70 subunits which are encoded on different genes.

5 Anti-Inflammatory Cytokines

Antiinflammatory activity is also reportedly increased in the elderly. IL-10 has both anti-inflammatory and B-cell stimulatory activities. It is produced by activated T-cells, B-cells, monocytes/macrophages and dendritic cells and is thought to block the ability of monocytes etc to act as antigen presenting cells by down-regulating the MHC. IL-10 is an important anti-inflammatory cytokine, capable of inhibiting the synthesis of proinflammatory cytokines such as IFNγ, TNFα, IL-2 and IL-3 and is produced in higher amounts by stimulated leucocytes from elderly subjects com-

pare to the young (Rink et al. 1998). IL-10 production by unstimulated monocyte monolayers was also found to be increased in a group of very elderly subjects and correlated with IL-6 production from the same monocytes (Rea et al. 1996a).

The GG 1082 allele of the IL-10 promoter polymorphism, a polymorphism associated with increased IL-10 production, was found to be increased in male centenarians compared to young controls, suggesting that an increased antiinflammatory state is the key to longevity in men (Lio et al. 2002). However, this was not found in Finnish or Irish nonagenarian studies (Rea et al. 2006). It also stands contrary to other findings where increased IL-10 production has not given survival advantage. Patients with meningococcal septicaemia who are high IL-10 producers have a 20-fold higher chance of a fatal outcome compared to low producers (Westendorp et al. 1997). Also children with sudden infant death tend to have high IL-10 levels or high IL-10 producer allele status (Summers et al. 2000). Therefore perhaps only homozygous GG 1082 men who have avoided serious bacterial infections earlier in life may have an increased chance of longevity. An antiinflammatory genotype might be advantageous later in life, when a chronic proinflammatory state appears to develop. This phenomenon is called Inflamm-ageing (Franceschi et al. 2000) and is more evident in males compared to females, which may explain the higher frequency of antiinflammatory genotype in very elderly males.

IL-19 is a relatively new member of the IL-10 family, whose full function remains to be elucidated. IL-19 induces the production of IL-10 and IL-19 from PBMCs (Jordan et al. 2005). It also stimulates production of IL-6 and TNFα from monocytes in vitro (Liao et al. 2002), suggesting it may exhibit pro-inflammatory activities. As increased production of both IL-6 and TNFα are reported in ageing, IL-19 may also play a role in the ageing process. To date there have been no reports of any age associated changes in IL-19 levels, however, a recent Japanese study of 500 subjects aged between 19 and 100 years has shown an association between IL-19 gene polymorphisms and age (Okayama et al. 2007).

TGFβ is another cytokine with anti-inflammatory activities which seems to have an important role in ageing. In a study of Italian centenarians the active cytokine was found to increase with age and there was a significant difference found for the genotype and allele frequencies at the +915 site on the TGFβ gene (Carrieri et al. 2004). This increase in the active anti-inflammatory cytokine may contribute to longevity by counteracting the harmful effects of the increased inflammatory activities seen in advanced age.

Cytokine antagonists also play a role in the anti-inflammatory response. IL-1 receptor antagonist (IL-1RA), produced by monocytes and macrophages, blocks the binding of IL-1 to its cell surface receptors. Also 2 distinct soluble forms of the TNF-receptor occur in the plasma of healthy individuals, where they bind TNF and act as physiological inhibitors of TNF activity (Seckinger et al. 1989). A study of elderly Italian subjects (mean age 79.6±5.8) found that plasma concentrations of both IL-1RA and sTNFr were greater in healthy aged subjects compared to young controls. Levels of plasma neopterin, a product of activated monocytes/macrophages, were also elevated and positively correlated with both IL-1RA and sTNFr, suggesting that the increase in these antagonists is due to monocyte activa-

tion in elderly people (Catania et al. 1997). Another Italian study of aged subjects (range 66–80 years old) and 20 centenarians also showed sTNFRI and sTNFRII to be significantly elevated in healthy old subjects compared to young controls, and even higher in centenarians (Gerli et al. 2000). Soluble CD30, another member of the TNF superfamily, was also increased in the plasma of centenarians compared to the young.

As cytokines do not work alone but are instead a part of a complex network, more studies are needed of the balance of the pro- and antiinflammatory cytokines in successful ageing. Lio et al. (2003) report that a combination of high IL-10 and low TNFα producer polymorphisms is a combination that favors longevity in males but not females. However, the number of males in the study was small. Further larger studies are therefore required into the balance of these systems in elderly subjects.

6 TH1/TH2 Cytokines

Helper T-cells (TH) in humans have been classified into either TH1 or TH2 cells depending on the cytokines they produce. IL-12, IL-2 and IFNγ are associated with TH1 responses while IL-10, IL-4, IL-6 and IL-13 are prominent TH2 cytokines (Mosmann et al. 1996). Altered cytokine production in elderly people has suggested that there is a shift towards a Type 2 cytokine profile. However, not all findings have been clear cut.

Most studies have shown that lymphocytes from elderly subjects produce significantly less IL-2, the most important T-cell growth factor, compared to the young (Caruso et al. 1996; Gillis et al. 1981; Rea et al. 1996b). Methodology of intracellular cytokine detection has shown no change in the proportion of IL-2+ve T-cells (McNerlan et al. 2002) or an increase (Pietschmann et al. 2003). IL-15, another stimulator, particularly of memory T-cells, has been found to be increased in the serum of centenarians compared to both young and old controls (Gangemi et al. 2005). Interestingly there was no significant difference between the young and the old. As IL-15 is an important stimulator of memory T-cell proliferation, this may explain the accumulation of memory T-cells in the very elderly individuals.

IFNγ is the major TH1 cytokine. Caruso et al. (1996) showed a significant decrease in both IFNγ and IL-2 production by mitogen-stimulated mononuclear cell cultures from elderly subjects but no significant difference in TNFα, IL-4 and IL-6. Rink et al. (1998) also reported that IFNγ is produced less by lymphocytes of elderly people.

However, several reports have shown, using intracellular cytokine detection methods, an increase in the percentage of IFNγ positive T-cells in aged subjects (McNerlan et al. 2002; Pietschmann et al. 2003; Sandmand et al. 2002). Sandmand and Rink also showed that IL-4 and IL-10 positive T-cells were increased in aged subjects. Pietschmann showed that some changes were gender-specific. In elderly women they showed an increase in the proportion of T-cells positive for IFNγ, IL-2,

IL-4, IL-10 and IL-13. In men they only saw an increase in IL-2, IL-4 and IL-13. Therefore changes in IFNγ and IL-10 seemed likely to be gender-specific.

Zanni et al. (2003) showed an increase in both type 1 (IFNγ, IL-2 and TNFα) and type 2 (IL-4, IL-6, IL-10) cytokines with age. Type 1 cytokine-positive cells in all three CD8+ subsets investigated (CD95-CD28+(naïve), CD95+CD28- (effector/cytotoxic) and CD95+CD28+ (memory)). An increase in type 2 producing cells was only seen in the memory CD8 cells.

7 Conclusions

Cytokine expression and production drives and modulates the inflammatory response through the complex network of activating and down-regulating interactions, always striving to achieve a homeostatic milieu after the "stress/danger" response, whether bacterial, viral or other, has been quenched.

As with other body systems, the homeostatic control, titration and modulation of immune responsiveness seems to become more fragile and less tightly focused with increasing age and this may explain some of the dissonance between the proinflammatory and anti-inflammatory control mechanisms and some of the elements of immune-ageing.

However, there is suggestive evidence that other factors both genetic and environmental, together with sex, are likely to have or have had an important influence on shaping the immune profile of our most aged people. In geographically separate populations, cytokine allele shift seems to have been shaped by different bacterial, viral or antigen exposure. Similarly in nonagenarians and centenarians, there is some suggestion of an allele frequency shift towards a more anti-inflammatory profile which may have a gender-weighted effect. It is not clear whether this is acquired or innate, or a "nature" or "nurture" effect. An interesting suggestion might be that survivors of the 1915 influenza pandemic, such as present-day nonagenarians, may carry a cytokine genotype profile which both facilitated their survival from the influenza epidemic but allowed survival to very old age, in an environment where antibiotic use could soften the need for an action-packed immune responsiveness.

Much research needs to be carried out to answer these very challenging but fascinating questions, which have an important role in helping us understand our immune systems better, the role which they have in protecting us from acute and chronic disease and improving the quality of our ageing. A large pan European study, such as is currently being carried out with the "platinum seniors" of Europe in European Union-funded Genetics of Healthy Ageing (GEHA) project, has the organizational breadth of ability and the statistical weight, to help answer some of these questions.

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Neuro

Neuro-Endocrine-Immune Network and its Age-Related Changes

K. Hirokawa and M. Utsuyama

Contents

1 Introduction

In living beings, external stimuli elicit a behavioral, verbal, or physiological response. The stimuli can be classified into 2 types: ordinary stimuli such as sound, smell, sight, and touch that are received by the sensory organs and pathological stimuli such as bacterial, viral, and fungal infections that are received by the immune system (Fig. 1).

Ordinary stimuli that are received by the sensory organs, perceived by the cerebral cortex, and recognized by the association cortex, stimulate the limbic system and finally reach the hypothalamus. In contrast, infections stimulate the cells of the immune system and induce the production of various cytokines that are transported via the blood stream to the brain and influence the function of the hypothalamus [1].

The hypothalamus has many centers that are responsible for various functions such as the regulation of pituitary secretion, sexual behavior, reproduction, water balance regulation, satiety, autonomic nerve regulation, feeding, circadian rhythms, aggressiveness, fighting behavior, drinking, exploration behavior, and heat conservation [2]. It is believed that the hypothalamus influences the functioning of the immune system via 1) pituitary-adrenal-gonad axis and 2) the innervation of lymphoid organs.

K. Hirokawa (✉)
Institute for Health and Life Sciences
Ascent Myogadani 4F, 4-4-22, Kohinata
Bunkyo-ku, Tokyo 112-0006, Japan
E-mail: hirokawa@h-ls.jp

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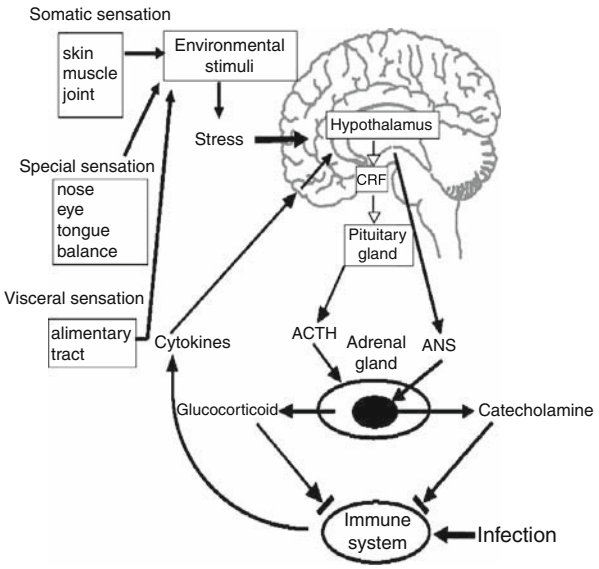

Fig. 1 Activation of the neuroendocrine-immune network by stress and infection. The various types of environmental stimuli that are received by sensory organs act as stress factors when they exceed normal physiological limits. This stress eventually stimulates the hypothalamus to secrete corticotropin releasing factor (CRF). CRF then stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete glucocorticoids. At the same time, the hypothalamus also stimulates,the autonomic nervous system (ANS), leading to the secretion of catecholamines by the adrenal medulla. Infections independently stimulate the immune system to produce various types of cytokines, most of which can enter the brain though areas where the blood-brain-barrier is weak, and stimulate the hypothalamus. This eventually results in the secretion of both glucocorticoids and catecholamines

Thus, all stimuli that are received by the body can reach the hypothalamus and influence its functions including the functions of the endocrine and immune systems. This consequently leads to the activation of the neuroendocrine-immune network.

Ordinary stimuli are essential for the normal development and growth of a living body. Physical stimuli help the development of normal body constitution. Visual and auditory stimuli including words and language promote the normal psychological and spiritual development of humans. Exposure to various infectious agents is also necessary for the normal development of the immune system.

It is not unusual for stimuli to exceed the normal physiological range. In such situations, these stimuli act as a source of stress for the body. In such cases, the hypothalamus plays a major role in the activation of the neuroendocrine-immune network for the maintenance of homeostasis. Homeostatic control involves the activation of the autonomic nervous system [3] and the hypothalamus-pituitary-adrenal axis. The former induces the production of catecholamines and the latter, glucocorticoids. These products are essential for the maintenance of homeostasis when the body is exposed to stress. However, both catecholamines and glucocorticoids have

a suppressive effect on the immune system. This is a kind of physiological trade-off. In any event, stress downregulates the activity of the immune system.

It is important to note that the nervous, endocrine, and immune systems change with age, and thus, the action of the neuroendocrine-immune system against stress also changes with age. To put it plainly, the ability of the neuroendocrine-immune system to cope with stress declines with age [1]. This chapter will briefly summarize various neuroendocrine-immune interactions and the age-related changes in these interactions.

2 Neuroendocrine-immune Interactions at the Cellular Level

Lymphocytes can produce various hormones and neurotransmitters [4,5] and express receptors for these molecules. Table 1 shows the common mediators released by the cells of the immune and neuroendocrine systems. The cells of the immune system produce many interleukins (ILs). It has become apparent that the cells of the nervous and endocrine systems can also produce most of these ILs and express receptors for them. The right hand side of Table 1 lists the mediators originally produced by the cells of the neuroendocrine system. It is now commonly accepted that the cells of the immune system produce pituitary hormones and express their receptors. Fig. 2 shows the expression of hormones and neurotransmitter receptors in the splenic T-cells of mice, as determined by reverse transcription-polymerase chain reaction (RT-PCR). It is interesting to note that the expression levels of these receptors change in a variable pattern with age. Expression levels of mRNA of the glucocorticoid and thyroid stimulation hormone (TSH) receptors do not change greatly with age. The levels of the thyrotropin-releasing hormone receptor (TRH-R), adrenocorticotropin

Table 1 Common Mediators

Immune system		Neuroendocrine system
		Endorphin, Encephalin
		Somatostatins, Substance P
Interleukins		Catecholamine, Acetylcholine
Interferons		VIP
		GH, TSH, PRL, ACTH
		LH, TRH, CRH, LHRH
		Thyroxin
		Insulin
		Adrenal steroids
		Gonadal steroids
	Serotonin*	
	Histamine*	
	Prostaglandin*	

Asterisk (*) indicates substances that were considered to be produced by both the immune and neuroendocrine systems.

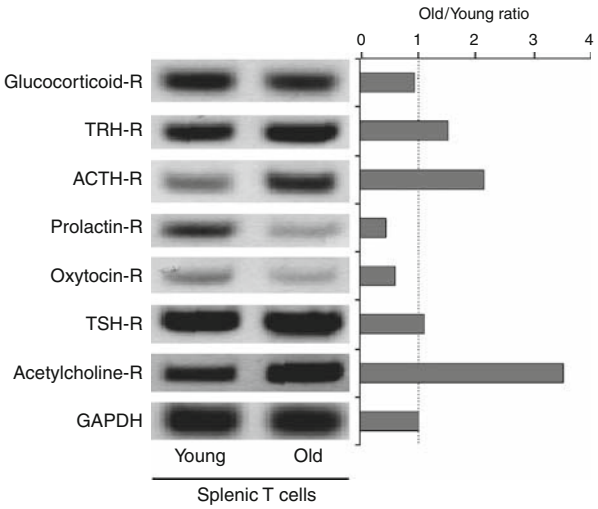

Fig. 2 The mRNA expression of various hormone and neurotransmitter receptors (R) in splenic T-cells from young and old mice. The columns indicate the ratio of old to young mice. The mRNA expression levels of thyrotropin-releasing hormone receptor (TRH-R), adrenocorticotropin receptor (ACTH-R), and acetylcholine receptor (acetylcholine-R) are increased in the T-cells from the old mice

receptor (ACTH-R), and the acetylcholine receptor (acetylcholine-R) increase with age while those of the prolactin receptor (prolactin-R) and the oxytocin receptor (oxytocin-R) decrease with age [1]. These facts suggest that interactions between the cells of the immune and neuroendocrine systems change with age.

3 Neuroendocrine-immune Interaction at the Organ Level

3.1 Hypothalamus-pituitary Axis and Immune System

The hypothalamus plays an important role in the control of both endocrine functions and the autonomic nervous system. Accordingly, it also operates as a control center for immune functions.

Thymic hypoplasia with T-cell-dependent immunodeficiencies was observed in Snell dwarf mice with congenital hypopituitarism [6]. This is consistent with the fact that the suppression of pituitary functions either by hypophysectomy [1] (Table 2) or the administration of antipituitary antibodies [7] results in a decrease in immune functions. Conversely, implanting growth hormone (GH)-producing cell lines in rats resulted in the reversal of the thymus atrophy and induced the thymus to regrow to a larger size [8]. The effects of GH in the thymus are mediated by insulin-like growth factor-1 (IGF-1), and thymic functions are actually under the control of

Table 2 Weight of organs after AHTL and hypophysectomy

Treatments	Thymus	Adrenal	Ovary	Hypophysis
Control +Sham-AHTL	314 ± 23	57 ± 3	99 ± 4	12 ± 1
Control +AHTL	423 ± 19	35 ± 3	61 ± 2	15 ± 1
Hypox +Sham-AHTL	128 ± 8	11 ± 1	12 ± 1	(–)
Hypox +AHTL	146 ± 15	12 ± 1	13 ± 1	(–)

Each group, 5 rats. Control group, sham operation of hypophysectomy.AHTL, lesioning of anterior hypothalamus. Sham-AHTL, sham operation of AHTL.Hypox, hypophysectomy. Data, mean ± 1 SEM.

GH/IGF-1-mediated circuits [9]. These findings taken together indicate that thymic size and function are dependent on the serum GH level.

Several reports have indicated that electronic lesions in the anterior hypothalamus resulted in a decrease in thymic weight [10,11], presumably by compromising pituitary function. Lesions in the ventromedial nucleus result in a significant decrease in pituitary and plasma GH levels [12]. We extended these earlier studies in rats by widening the area of destruction in the anterior portion of the hypothalamus, including the anterior hypothalamic nucleus, suprachiasmatic nucleus, and periventricular nucleus, (hereafter referred to as anterior hypothalamic lesioning, (AHTL)) and performed AHTL not only in young rats but also in aged rats with an atrophic thymus [1,13,14].

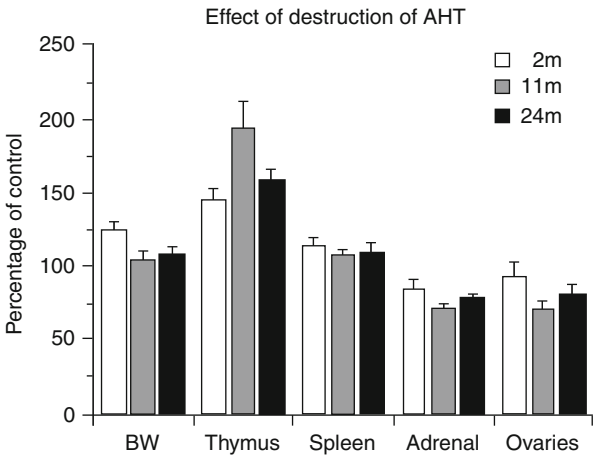

Fig. 3 Effect of destruction of the anterior portion of the hypothalamus (AHTL) in Wistar rats. AHTL was performed in rats at 2 months (open columns), 11 months (grey columns), and at 24 months of age (solid columns). Body weight and the weight of various organs were assessed 4 weeks after AHTL. The results are expressed as a percentage of the control age-matched rats that underwent a shamoperation. Vertical bars, 1 standard error of the mean

Contrary to the results of previous reports, AHTL resulted in thymic hypertrophy not only in young rats but also in middle-aged and old rats (Fig. 3). Interestingly, the magnitude of thymic hyperplasia after AHTL differed with age, indicating that the relationship between the hypothalamus and thymus changes during the course of aging. Furthermore, it was found that thymic hyperplasia did not occur in hypophysectomized rats (Table 2); thus, indicating that pituitary hormones regulate thymic hyperplasia.

Significant atrophy of the adrenal glands and gonads (testes or ovaries) was observed in rats subjected to AHTL, suggesting that ACTH and luteinizing hormone (LH) were not associated with thymic hyperplasia. High serum GH levels were noted in rats treated with AHTL, and these high levels were observed not only in young rats but also in middle-aged and old rats (Table 3). Since the secretion of GH is episodic, the single point sample data shown in Table 3 must be interpreted with caution. However, the rise in serum GH levels was consistent with the slightly hypertrophic pituitary gland in rats subjected to AHTL, i.e., the weight of the pituitary gland in the control and AHTL groups was 12 ± l mg and 15 ± 1 mg, respectively. Furthermore, high serum GH levels were not observed in adrenalectomized (adx) or ovariectomized (ovx) rats (Table 3).

We presume that a high serum GH level is necessary for thymic hyperplasia. When the serum GH levels of rats and mice at various ages were assessed, high serum GH levels were observed only at the newborn stage (Table 4). This is consistent with the results of a previous study [15] that reported profuse GH secretion in neonates. The fact that atrophy of thymus can be reversed and the thymus can be induced to regrow to a larger size by the administration of GH (8) suggests that the serum GH level shows a gradual decline with age. Actually, the fall in GH over

Table 3 Serum GH and LH levels in various experiments

Age	Treatments	GH(ng/ml)	LH(ng/ml)
6 weeks	Sham-AHTL	9.4 ± 2.2	6.8 ± 1.5
	AHTL	182.3 ± 7.0	4.0 ± 1.5
	Hypox + Sham	4.0 ± 0.9	2.4 ± 0.3
	Hypox + AHTL	3.1 ± 0.1	2.5 ± 0.6
2 months	Ovx	12.7 ± 1.7	14.1 ± 0.5
	Sham-Ovx	13.8 ± 1.4	3.7 ± 0.3
	Adx	15.6 ± 2.6	3.0 ± 0.1
	Sham-Adx	15.1 ± 4.6	3.3 ± 0.5
11 months	Sham-AHTL	26.9 ± 7.0	2.5 ± 0.2
	AHTL	176.0 ± 7.5	3.4 ± 0.3
24 months	Sham-AHTL	41.0 ± 18.4	2.8 ± 0.3
	AHTL	168.0 ± 0.7	3.0 ± 0.2

AHTL: lesioning of anterior hypothalamus. Sham-AHTL, shamoperation of AHTL. Ovx, ovariectomy. Sham-Ovx, sham operation of Ovx. Adx, adrenalectomy. Sham-Adx, sham operation of Adx.

Data, mean ± 1 SEM, obtained from 5 rats.

Table 4 Serum level of GH in mice and rats at various ages

Age	Rat	Mouse
18 fd	1.5	ND
NB	129.3 ± 5.4	141.7
1 month	3.9 ± 0.4	2.2 ± 0.6
3 months	14.0 ± 4.9	1.8 ± 0.4
6 months	12.3 ± 0.7	2.3 ± 0.3
12 months	10.4 ± 1.2	2.6 ± 0.1
18 months	8.8 ± 0.8	2.4 ± 0.2
24 months	12.0 ± 1.2	6.8 ± 2.5

Fd, fetal day. NB; Newborn Data: mean concentration of GH (ng/ml) ± SEM,obtained from 4 to 6 animals. Asterisk (*) indicates that the GH levels in samples pooled from several animals were assessed. ND, not done.

the life span is from 1200 μgm^{-2} in adolescents to 60 μm^{-2} in older individuals [15]. However, the most important point to be noted is that GH secretion appears to be extraordinarily high at the newborn stage in mice, rats, and humans and that this high level is necessary for thymic growth.

These findings collectively indicated that the serum GH level is dependent on the balance between positive and negative signals (growth-hormone-releasing hormone (GHRH) and growth-hormone-release-inhibiting hormone (GHRIH), respectively) or somatostatin (SST) in the hypothalamus. We examined the mRNA levels of these positive and negative signals in the mouse hypothalamus and found that the level of GHRH mRNA decreased with age while that of pre-pro-SST mRNA increased with age. In addition, we also analyzed the receptors for these signals in the pituitary glands and found that with age, the level of GHRH-receptor mRNA also decreased while that of the SST receptor increased (Utsuyama, personal communication). These observations are consistent with those of some previous reports. Florio et al. [17] reported that the pre-pro-SST mRNA levels in the hypothalamus of 25-month-old rats were slightly greater than those in younger rats. Furthermore, an age-related increase was observed in the levels of the SST receptor (sst2) in the pituitary gland of aging rats [18]. Based on the fact that high levels of serum GH are observed only at the newborn stage in rats and mice (Table 4), it can be assumed that hypothalamic positive signal is superior to hypothalamic negative signal, resulting in a high level of GH. However, at later stages of development, the negative signal becomes superior to the positive signal, leading to a decline in the secretion of GH (Fig. 4). This concept has been validated in aging humans; i.e., available clinical data have suggested that excessive SST release occurred with diminished GHRH secretion [19]. Thus, the destruction of the anterior portion of the hypothalamus, which contains the cells that produce SST (negative signal), shifts the balance between the positive and negative hypothalamic signals toward the predominance of the positive signal. This results in high serum GH levels even in the middle-aged and old rats, and eventually leads to thymic hyperplasia.

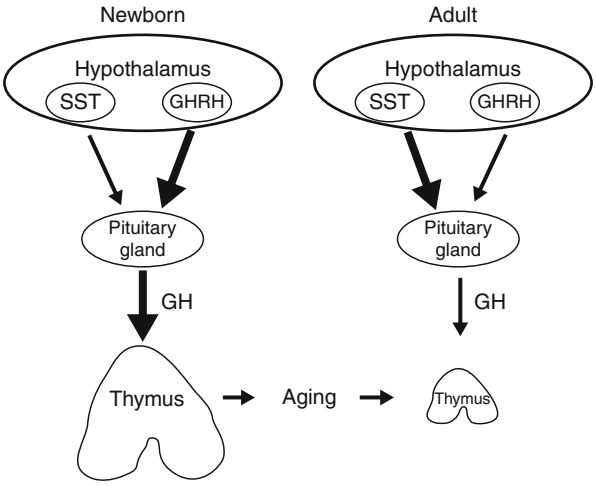

Fig. 4 Schematic representation of the control thymic function by the hypothalamus-pituitary axis. In newborn animals, the strength of the positive signal of growth hormone releasing hormone (GHRH) is greater than that of the negative signal of somatostatin (SST) in the hypothalamus, leading to an increased secretion of growth hormone (GH). In young adult animals, the strength of the negative signal is greater than that of the positive one, leading to a decrease in the secretion of GH. A decrease in the serum level of GH in the young adult results in to thymic involution

In addition to having various centers for the control of endocrine functions, the hypothalamus is closely related with the sympathetic nervous system (SNS). As the thymus is rich in nerve fibers [20], it is possible that the hypothalamus also influences thymic function through the sympathetic nerve fibers. To test this possibility, we examined the effect of AHTL on both the host thymus and the thymus implanted under the kidney capsule. Briefly, 2 lobes of a newborn thymus were implanted under the kidney capsule in young rats, and AHTL was performed on these rats after 1 month. One month after AHTL, the rats were sacrificed and the weight of the host and implanted thymuses was assessed. Contrary to our expectation, AHTL had no effect on the implanted thymus; although the host thymus, however, became hyperplastic. However, hypophysectomy greatly influenced both the host and implanted thymuses (Table 5). These results suggested that thymic hyperplasia after AHTL depends not on only the high serum GH levels but also on some unknown local requirement. The variation in the response could be attributed to the difference in autonomic innervation between the host and the implanted thymuses. The stimulation of the SNS is known to suppress immune function. Miles et al. [21] reported that ablation of the peripheral nervous system caused a significant increase in splenic T-cells. Earlier studies by Besedovsky et al. [22] reported that surgical denervation of the rat spleen resulted in an increase in the antibody-forming activity. Therefore, it is likely that AHTL affects the functions of the SNS, and alterations in the SNS around the host thymus might be essential for the development of thymic hyperplasia after AHTL in addition to the high serum GH levels. Furthermore, a decrease in serum GH levels by hypophysectomy leads to a significant atrophy of both the implanted and host

Table 5 Effect of AHTL on the host and implanted thymuses in normal and hypophysectomized rats

Treatment	Host thymus	Implanted thymus	Adrenal gland
Sham-AHTL	304 ± 23	137 ± 11	57 ± 5
AHTL	441 ± 13	102 ± 21	36 ± 3
AHTL + Hypox	105 ± 11	47 ± 12	19 ± 5

A new-born thymus was grafted under the kidney capsule in 2-month-old normal rats or in hypophysectomized rats. One month after the implantation of the thymus, the rats underwent AHTL, and 1 month later, they were sacrificed for the assessment of organ weight. AHTL, lesioning of anterior hypothalamus. Sham-ATHT, sham operation of AHTL. Data, mean ± 1 SEM, obtained from 5 rats.

thymuses (Table 5). Further experiments are clearly necessary to clarify the relationship between nerve fibers, the thymus, and the hypothalamus.

3.2 Adrenal Glands and the Immune System

With the exception of erythrocytes, most cells have glucocorticoid receptors. Therefore, physiological and pharmacological effects of this hormone are very variable. Glucocorticoids are known to have distinct antiinflammatory, immunosuppressive, and oncostatic effects. Glucocorticoid immunosuppression is mediated by a direct cytolytic effect, through the inhibition of lymphocyte function, or indirectly through soluble suppressor mediators [23]. In vivo glucocorticoid administration results in pronounced thymic involution, and immunohistological analysis of the changes following glucocorticoid performed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method reveal the development of extensive apoptosis in the cortex [24].

Adrenal glands removal in mice leads to a significant increase in the weight of the thymus and spleen as well as in the number of splenic T-cells (Table 6). The effect is partly due to a decrease in the serum levels of glucocorticoids and partly due to an increase in the serum levels of the adrenocorticotropic hormone (ACTH) in the serum. These changes are mediated by a negative feed back reaction through the hypothalamic-pituitary axis. Interestingly, the thymus can also influence the function of the adrenal glands, i.e., it was observed that the implantation of a newborn thymus increased the weight of the adrenal glands in nude mice [25]. This increase in weight

Table 6 Effect of adrenalectomy on thymus and splenic T-cells in rats

Groups	Body weight	Thymus	Spleen	Splenic T cells
Sham	194 ± 4	233 ± 4	195±7	1.49 ± 0.12
Exp	192 ± 3	417 ± 19	549 ± 24	2.33 ± 0.44

Exp indicates adlenalectomy.

might be mediated by the secretion of ACTH by the T-cells following appropriate stimulation [26].

3.3 Gonads and the Immune System

Sex steroids are known to suppress immune functions [27]. We also reported that various steroids suppressed the in vitro proliferation of mouse spleen cells by mitogenic stimulation [28]. Physiological thymic involution that starts around puberty can be ascribed to the increased level of sex steroids. Interestingly, this thymic involution is not an irreversible phenomenon. In mice and rats, thymus atrophy at any age can be

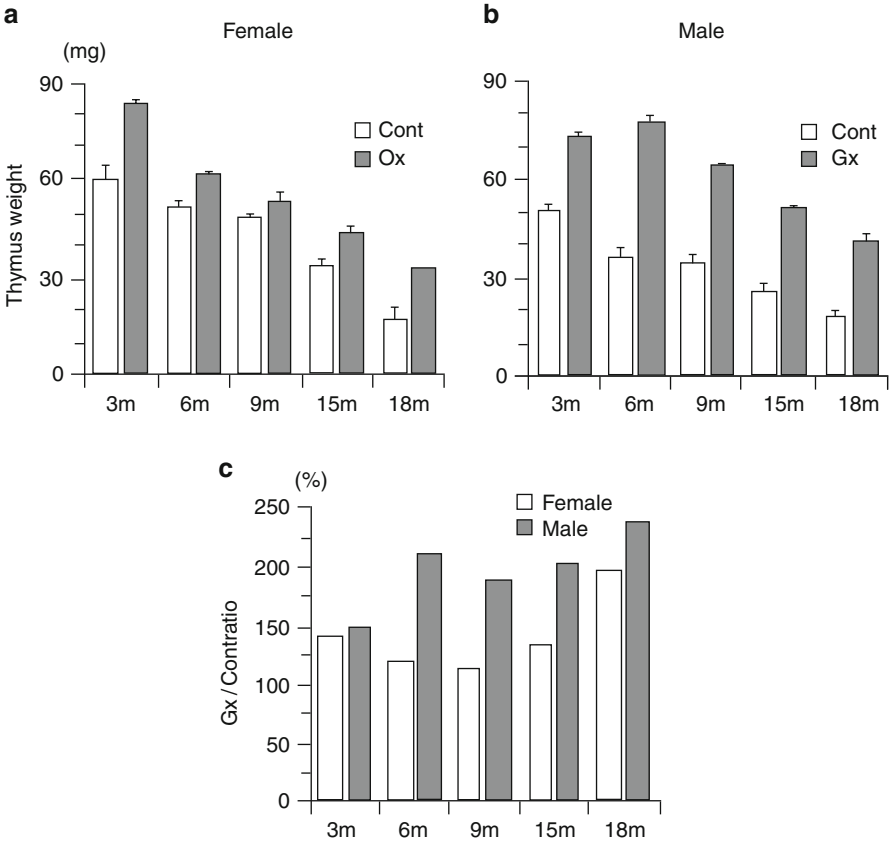

Fig. 5 Effect of gonadectomy on thymic weight in female (a) and male (b) C57BL/6 mice. Thymic weight was assessed 2 months after gonadectomy. Open columns, sham operation (Cont). Grey columns, gonadectomy (Gx). (c) Ratio of gonadectomized to control mice (Gx/Cont ratio). The magnitude of increase in thymic weight after Gx is more prominent in males than that in females, and thymic weight tends to increase with age

reversed, and the thymus can be induced to regrow to a larger size by the removal of testes or ovaries (Fig. 5a, Fig. 5b) [29–32]. It is important to note that the extent of thymic restoration after gonadectomy in our study was gender- and age-dependent. The size of the restored thymus was considerably larger in males than in females and in older mice than in younger mice (Fig. 5c). The restoration of thymic size after gonadectomy is temporary and is observed for several weeks. It differs from the long-term thymic hyperplasia observed after AHTL. Thymic restoration after gonadectomy may be simply due to the decreased suppressive effect of sex steroids on lymphocytes [30]. The decrease in the serum levels of sex steroids stimulates the hypothalamus-pituitary axis through negative feedback to secrete hormones capable of restoring thymic size and cellularity. This concept has been supported by the fact that thymic hyperplasia after gonadectomy does not occur in hypophysectomized rats [31].

4 Neuroimmune Interaction at the Time of Infection [33]

Overwhelming evidence suggests that various cytokines and their receptors are present in the brain and influence its functions. It was previously thought that large molecules such as cytokines are prevented from entering the brain by the blood-brain-barrier (BBB). However, it has been clearly shown that recombinant IL-2 injected into patients can enter the brain through areas where the BBB is weak, and exert a neuromodulatory effect [34].

Lipopolysaccharide (LPS) is known to trigger an acute-phase response and the synthesis of proinflammatory cytokines. LPS injection in experimental animals induces various neurological manifestations and physiological changes such as fever, hypotension, and the secretion of variable hormones and is therefore used to develop infection models.

During an infection, cytokines are mainly produced in the immune system and partly, to some extent, in the brain. Therefore, it is quite likely that cytokines produced in the immune system might influence the neurological and physiological functions of the brain.

It is interesting to note that the mRNA level of various cytokine receptors was found to be increased in the spleen of young mice but not old mice. A similar enhancement in the levels of cytokine receptor mRNA was also observed in the brain of mice after LPS stimulation, but the magnitude of this increase varied according to the type of cytokine receptor, the brain region, and the age of the mice [33].

Fig. 6 indicates the mRNA expression level of various cytokine receptors in the cerebral cortex after LPS injection in mice. In young mice, the mRNA levels of IL-1R1, IL-2Ra, IL-3R, and IL-6R peaked at 3 or 6 h after LPS injection. In old mice, the expression of IL-1R1 and IL-3R was delayed, definitely lower in IL-2Ra, and almost similar in IL-6R. However, the mRNA expression level of tumor necrosis factor (TNF)αR and interferon (IFN)γR was higher in the old mice than in the young mice.

In any event, cytokines produced by immune cells might directly or indirectly influence brain function through the various cytokine receptors expressed in the brain. Moreover, the interaction between the immune system and the brain during

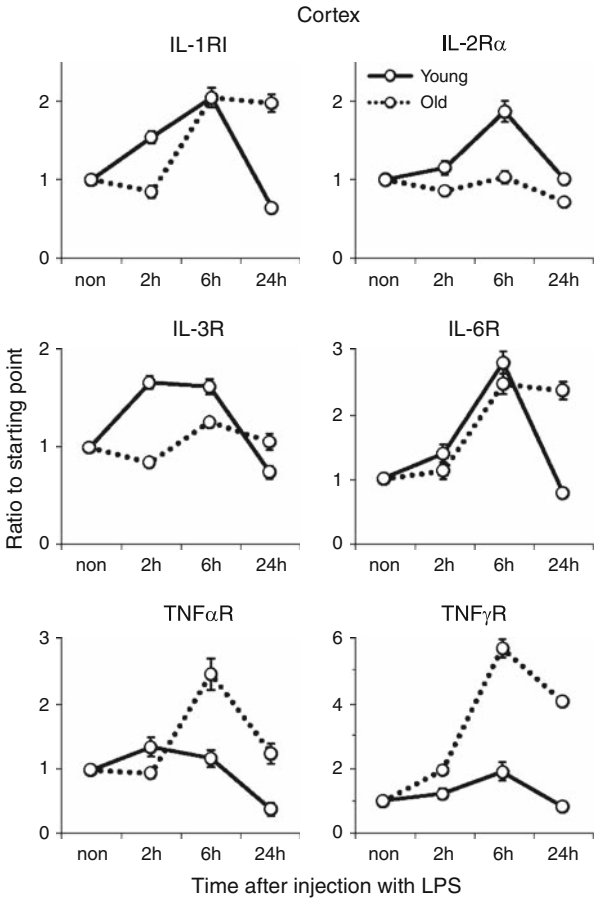

Fig. 6 Changes in the mRNA level of various cytokine receptors in the frontal cortex of mice after lipopolysaccharide (LPS) injection (30 mg/mouse) The type of cytokine receptor is indicated in the upper right-hand corner of each graph. Open circles with solid line, young mice. Open circles with dotted line, old mice. Each point, average of 3 samples. Vertical bars, standard error of the mean. Ordinate, the ratio of the mRNA level after LPS injection to that before the injection

an infection is expected to be different in young and old mice because cytokine production changes with age as does the expression of cytokine receptors in the brain [35]. It is interesting to note that the mRNA levels of some cytokine receptors in old mice were higher than that in young mice after LPS stimulation. In other words, the neuroimmune interactions are subject to change with advancing age, and these changes could be responsible for the fact that the elderly are vulnerable to various physiological disorders during an infection.

5 Conclusion

All environmental stimuli including infection eventually reach the hypothalamus and influence its function. The hypothalamus has many centers, which are essential for the maintenance of the life. An important hypothalamic function is the control

of the neuroendocrine-immune network. Therefore, all environmental stimuli are processed by the neuroendocrine-immune network. When the stimuli exceed the normal physiological range, the neuroendocrine-immune network plays a major role in the maintenance of the internal environment, which is known as homeostasis. With aging, however, the activity of the 3 systems, i.e., the nervous, endocrine, and immune systems, decline and so does the homeostatic capacity of the neuroendocrine-immune network. This chapter has shown several examples of age-related changes observed in the neuroendocrine-immune network at both the cellular as well as the organellar level. 1) The mRNA expression levels of hormone and neurotransmitter receptors in T-cells changes with age. 2) The destruction of the anterior portion of the hypothalamus causes thymic hyperplasia, and the extent of this hyperplasia varies with the age of the animal,. 3) Thymic involution is controlled by the hypothalamus. 4) Gonadectomy has a serious influence on thymic weight, and its effect varies with gender and age. 5) An intravenous injection of LPS induces an elevation in the mRNA levels of the receptors of various cytokines in the brain. The extent of this elevation varies with age and the brain region investigated. These findings indicate that the homeostatic control of the neuroendocrine-immune network is more efficient in young than in old individuals.

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Sex Hormones and Immunosenescence

Christian R. Gomez, Vanessa Nomellini and Elizabeth J. Kovacs

Contents

E. J. Kovacs (✉) · C. R. Gomez · V. Nomellini
Loyola University Medical Center
Bldg. 110, Room 4232
2160 South First Avenue
Maywood, IL 60153, USA
Phone: 708.327.2477
Fax: 708.327.2813
E-mail: ekovacs@lumc.edu

C. R. Gomez
Facultad de Ciencias de la Salud
Universidad Diego Portales
Ejército 141, Santiago, Chile

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Abstract: Sex hormones impact a number of aspects of immunity. As a result of the aging process, dramatic changes occur in the endocrine system, including the levels of sex hormones. Thus, it is possible that the hormonal environment may play a role in the effects of aging on normal aspects of the aging immune system, as well as in immune responses to injury and infection. Although much has been discovered regarding age-related changes in the immune and the endocrine systems, the exact mechanism of the interplay between these factors has yet to be resolved. In this chapter, we explore each of these areas and investigate how sex hormones may be an important component to immunosenescence. Finally, both beneficial and adverse effects of hormone replacement therapy on the aging process are discussed. As age and gender are potential modifiers of the disease process, therapies targeted to the specific hormonal and immune status of an individual may prove to be most beneficial for optimal clinical outcomes.

1 Introduction

In studying human longevity, two observations clearly stand out: first, the highest increase in life expectancy recorded in history has occurred in the past century and [186], second, the average lifespan of women is almost 10% higher than men [227]. The fact that this sex difference occurs not only in humans but also in many mammalian species suggests that the hormonal environment plays a role in the aging process.

Among the prominent effects of sex on aging is the response of the immune system. While not as dramatic as the role that sex steroids (estrogens and androgens) play in sexual differentiation and reproduction [188, 248], their effects in immune function have been well documented [48, 188, 248]. Overall, females exhibit stronger humoral and cell-mediated immune responses than males [248]. For example, females in their reproductive years have higher plasma levels of immmonoglobulins (Ig), such as IgM and IgG, and mount more vigorous antibody responses than males after immunization or infection [reviewed in [10]]. Females also exhibit a more rapid allograft rejection compared to males [93]. As a result of this heightened immunity, females also have an increased susceptibility to various autoimmune diseases. Additionally, female to male incidence of developing of rheumatoid arthritis is 2–4:1, 5–13:1 for systemic lupus erythematosus and 25–50:1 for Hashimoto thyroiditis [10]. There are also sex differences in the response to injury [9, 96]. Among those sustaining most types of traumatic injury, male patients show increased mortality compared with female patients [82]. In addition, females have significantly higher infection and sepsis survival rates [33, 188, 215], and a lower risk for postinjury pneumonia [80]. Interestingly, unlike other forms of traumatic injury, females have greater mortality following burn injury than males [127]. This may be sex hormone mediated during reproductive years, but since it occurs over most of the life span, from ages 10–70 [127], factors other than sex hormones alone are likely to be involved.

While chromosomal effects may explain some of the sex differences in immunity, the hormonal environment seems to have the greatest influence [10]. As part of the normal aging process, changes occur in both the immune and endocrine systems. Since the endocrine system is an important component in the regulation of the immune system, it is possible that a more complex interplay between these two systems exists. Understanding the consequences of aging on immunity is further complicated by genetic background, mutations, oxidative damage, etc. [232]. In this chapter, we will first describe how sex hormones modulate the immune response. Next, we will examine how the endocrine system changes with age, focusing on the sex hormones. We will then describe specific manifestations of immunosenescence from a perspective involving age-associated changes in sex hormones. Finally, the therapeutic and adverse effects of sex hormone replacement on the aging process and on specific aspects of immunosenescence will be discussed. Although progress has been made with regard to the effects of age on the immune system and the endocrine system, the exact mechanism of the interaction between these systems in the elderly has yet to be resolved. In this review, we will explore each of these areas and investigate how sex hormones may be an important component of immunosenescence.

2 The Effects of Sex Hormones on the Immune Response

During reproductive years, females have a more robust humoral and cellular immune response compared to males [98]. Depending on the concentration of estrogen, it can either be immunostimulatory or immonosuppresive. *See* Figure 1 for the biosynthetic pathway of the sex hormones. At levels seen over the menstrual cycle (in particular proestrous levels of estrogen) boost immunity. However, in pregnancy higher levels of estrogen are immunosupresive [41, 233]. In contrast to estrogen, all concentrations of testosterone are though to be immunosupresive [35, 177]. Sex hormones modulate immune cell responses through direct and indirect actions on a series of targets, including lymphoid organs, T cells, B cells, natural killer (NK) cells, and macrophages. For instance, the number of $CD4^+$ lymphocytes is higher in females [178] and thymocytes and lymphocytes from female mice respond more vigorously to antigens than those from males [255]. In addition, the production of cytokines, such as interleukin (IL)-1β was higher in macrophages from females after in vitro stimulation [106]. In addition, the production of IL-4 [60] and interferon-gamma (IFN-γ) [214] was higher in splenocytes from females compared to males. Sex hormones also influence the immune system through their actions on the central nervous system, bones, endocrine organs, and nonlymphoid tissues (liver, kidney, complement producing cells, and mucosal epithelial cells) [48].

The immunomodulatory role of estrogen, particularly on lymphopoiesis and immune responses have been studied extensively (reviewed in [147, 233]). Periovulatory levels of estradiol have been shown to stimulate antibody production by

B cells [50, 76, 172, 190]. However, this increases the potential for autoimmune diseases [2, 182, 247]. In contrast, peri-ovulatory estradiol levels led to a suppression of B cell lineage precursors [111, 165]. Pregnancy levels of estrogen, on the other hand, suppressed the T cell-mediated delayed-type hypersensitivity (DTH) reaction [43, 64, 95] and inhibited the release of tumor necrosis factor-α (TNF-α). These high levels of estrogen also stimulated T cell-induced IL-4, IL-10, and IFN-γ secretion [123, 211]. In macrophages, late pregnancy levels of estrogen inhibited LPS-stimulated IL-6 secretion [56, 112] and TNF-α release [239, 268]. In addition, secreted IL-1β levels were increased at peri-ovulatory levels, but inhibited at high pregnancy levels [201, 221]. This biphasic effect of estrogen is especially relevant when considering proinflammatory diseases in pre and postmenopausal women, as will be discussed later.

Progesterone is a major gonadal hormone synthesized primarily by the corpus luteum, the testes, and the adrenal cortex [217]. Besides its well-known endocrine and neuroprotective effects [217], progesterone has been suggested to have an immunosuppressive role. This is thought to play a protective role in pregnancy [229]. The regulatory effects of progesterone on the immune system include blocking cytotoxic T cell activity [159], reducing NK cell activity [102], and modifying the cytokine response [46, 197, 198].

Testosterone also has many immunomodulatory roles [35, 177]. T cell apoptosis is decreased in males compared to females, as reflected in the decreased numbers in the periphery of men [164]. In B cells, testosterone inhibited IgG and IgM secretion [121]. In contrast, endotoxin-stimulated monocytes from males produced more TNF-α than females [14, 34, 218]. Whether this response to endotoxin is due to increased testosterone concentrations remains uncertain, though, since in vitro studies have not shown an effect of testosterone on TNF-α production [202]. In vivo and in vitro analysis of immune-endocrine interactions, including manipulation of testosterone levels through castration, have elucidated the differences between males and females in terms of immunocompetence [177]. These differential roles of sex hormones on the immune system have been proposed to be main determinants of male versus female responses to injury and infection. As a result of the aging process, dramatic changes occur in the endocrine system, including the sex hormones. Thus, it is possible that the hormonal environment may play a role in the effects of aging on the immune system.

3 Endocrine Changes with Aging

3.1 Overall Changes

As a normal part of aging, hormonal changes occur resulting from a decline in secretion of hormones and/or availability of target cells. Perhaps the most char-

acterized hormone changes with age are the decline in secretion of estrogen in the ovaries (menopause) and testosterone in the testes (andropause) (all of the following hormonal changes with age are reviewed in [13, 44, 146]). As a result, the levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) released by the pituitary gland are elevated, due to the lack of negative feedback by the gonadal hormones. Changes in the sex hormone environment occurring as a result of advanced age are summarized in Table 1. Other changes in the endocrine system seen with age include a decreased release of growth hormone causing a diminished production of insulin-like growth factor-1 (IGF-1) by the liver and other organs (somatopause) and a diminished production of the sex hormone precursor, dehydroepiandrosterone (DHEA) by the adrenal cortex (adrenopause). Altogether, both central (hypothalamic and pituitary) and peripheral (ovarian, testicular, and adrenal) components of the endocrine system are affected over time and have thus been linked with the aging process [146].

Table 1 Sex hormone environment in advanced age

Factor	Females	References	Males	References
Tropic hormones				
FSH	↑	[116, 223]	↑	[171]
LH	↑	[116, 223]	↑	[171]
Sex hormones				
Pregnenolone	↓	[103]	↓	[103, 174]
Estradiol	↓↓	[92, 107, 156,180]	↓	[73, 100, 242]
Progesterone	↓	[79, 107, 223, 228]	no change or ↓	[29, 187]
Testosterone	↓	[54, 145]	↓	[36, 61, 120, 166, 252]
DHT	↓	[145, 224]	no change or ↓↑	[52, 70, 94, 246]
Androsterodione	↓	[94]	↓	[94]
DHEA (S)	↓	[94, 124, 149, 189]	↓	[94, 124, 149, 189]
Sex hormone receptors				
Estrogen receptors	tissue specific	[37, 114, 222, 258]	↓	[222]
Progesterone receptors	tissue specific	[37, 45, 78, 226]	tissue specific	[37, 45, 78, 225]
Androgen receptor	↓	[238]	↓	[210, 261, 270]
Others				
SHBG	no change or ↓↑	[40, 54, 161]	↑	[241, 242]
Aromatase	↑	[109, 110, 204]	↑	[109, 241]
5 α-reductase			↓	[241]

Arrows indicate increase or decrease in aged subject relative to young. FSH follicle stimulating hormone, LH luteinizing hormone, DHT dihydrotestosterone, DHEA (S) dehydroepiandrosterone (sulfate), SHBG sex hormone binding globulin.

4 Menopause and Andropause

In women, the average onset of menopause is 51 years of age, and results in a postreproductive period that encompasses nearly a third of their lives [116]. For most women, menopause is accompanied by vasomotor symptoms, depressed mood, changes in body composition (such as increased body fat), and an elevated risk of coronary heart disease, myocardial infarction, and stroke [116]. The use of animal models has helped to uncover some of the mechanisms involved in reproductive aging like humans, female primates exhibit hormone cyclicity in that extensive menstrual bleeding and shedding of the endometrial lining occur similar to humans [260]. However, utilization of humans and nonhuman primates for experimentation purposes involves a series of complications, including their extended lifespan and excessive research cost [260]. As an alternative, rodent models have been used. The estrous cycle in female rodents can be divided into four stages: proestrus, estrus, metestrus, and diestrus, with ovulation normally occurring during estrus [148]. In female mice, advanced age correlates with progressively longer estrous cycles, characterized by lower levels of estrogen [181]. Eventually, this decline in estrogen leads to the absence of ovarian follicle development and low plasma estrogen and progesterone concentrations [181]. The effect of aging on the estrous cycle in female rats is different from mice, in that they have well defined estrous cycles [157]. However, irregular cycles emerge at middle age, in which ovulatory activity occurs at longer intervals. This period is chronologically followed either by constant estrus, irregular pseudopregnancies, and anestrus [72, 157]. Overall, the decline in ovarian function differs between rats and mice, as well as between strains, and may occur between 6 and 18 months of age, or even up to 24 months for some strains [72].

Andropause, on the other hand, is defined as the progressive decline (0.8–2% each year) in testosterone levels, beginning at middle-age [175]. Unlike women, men do not have a universally recognized "syndrome of andropause," as the decline occurs more gradually [195]. The clinical features associated with andropause include increased body fat, loss of muscle and bone mass, fatigue, depression, anemia, poor libido, erectile dysfunction, insulin resistance, and a higher risk of cardiovascular disease [124]. Andropause is also present in male rodents; however this is strain dependent in both mice [36, 61] and rats [120, 166, 252].

5 Specific Changes in Sex Hormones with Age

5.1 Pregnenolone

Pregnenolone is the precursor of all known steroid hormones [194]. In humans maximum serum pregnenolone levels are achieved between 25 and 30 years of age in both men and women [103]. After this time, women exhibit a gradual decline

[103], while men maintain constant levels up to approximately 52 years of age, followed by a continuous decrease [103, 174]. Since circulating pregnenolone is mostly, if not entirely, of adrenal origin [103], these results have raised the question of the contribution of the adrenal glands to the defective production of sex steroids during aging [103].

5.2 Estradiol

The most dramatic change with the onset of menopause in women is an abrupt decrease in circulating estradiol. By perimenopause, serum estrogen concentrations decline, FSH concentrations become augmented to levels higher than in younger women, but LH does not change [223]. Eventually, follicular activity ceases, estrogen concentrations fall, and LH and FSH rise above premenopausal levels [116]. After menopause, however, small quantities of estrone—an estradiol precursor synthesized from androsterodione in the cortex of the adrenal gland and in interstitial ovarian cells—are converted to estradiol (Fig. 1). Thus, estradiol is still present, but the normal cycling levels seen prior to menopause are replaced

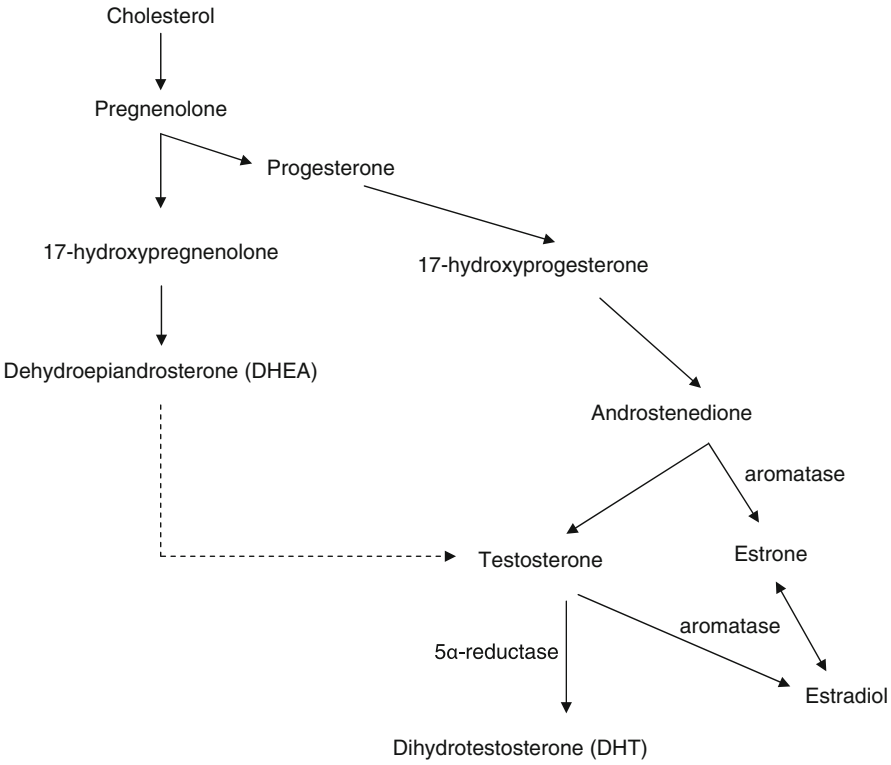

Fig. 1 Biosynthetic pathways for sex hormones

by levels that are much lower and do not fluctuate [156]. Similarly, during the period of lengthening cycles in female mice, a fall in circulating levels of estradiol can be measured [180]. In the phase of persistent diestrus, low plasma levels of estradiol were observed in conjunction with follicle-depleted ovaries [92], suggesting that ovarian aging in mice also contributes to the reproductive defect in estradiol production. Aged female rats, however, produced levels of estradiol at constant estrous levels, which are comparable to that of animals with regular cycles [107]. Since the ovaries of aged rats are capable of normal function under appropriate gonadotrophic stimulation, it has been hypothesized that altered hypothalamo–pituitary function is a major cause for cessation of regular estrous cycle in the female rat [107].

Estrogens in males, predominantly produced by peripheral aromatization of testicular and adrenal androgens, have diverse roles including spermatogenesis [185], sexual behavior [185], and development and maintenance of the skeleton [244]. As in women, serum concentrations of estradiol decrease in men [73]. This decrease in males has been attributed to a decline in free estradiol, or that which is unbound to sex hormone-binding globulin (SHBG)—the carrier protein used by estradiol and testosterone in the serum. Levels of the SHBG-bound fraction of estradiol, in contrast, increase with age [242]. With this estradiol reduction in men, a more pronounced decline in estrone is also observed [242]. Studies in rodents, on the other hand, have shown interesting results. Aged male Fisher 344 rats, which frequently have Leydig cell hyperplasia or develop testicular Leydig cell tumors, had augmented levels of estradiol, mainly at the testicular level [99]. On the contrary, male Brown Norway rats exhibited decreased circulating levels of estradiol with age, and orchidectomy produced a progressive decline in FSH and LH [100]. These findings suggest that aged male Brown Norway rats experience both primary and secondary testicular failure. Therefore, this strain is likely a better rat model for studying male reproductive aging, compared to Fisher 344 rats.

5.3 Estrogen Receptors

Estrogen receptor (ER)α and ERβ belong to the steroid/thyroid hormone superfamily of nuclear receptors [183]. These receptors are expressed in a variety of immune cells, including T cells and macrophages [233]. The decline in circulating estrogen associated with advanced age may also differentially modulate ER levels in males and females. ERα mRNA and protein levels were decreased and ERβ was virtually absent in uteri from aged mice [222]. Similarly, sex, age, and region-dependent expression of ERα and ERβ were found in rat brains [37,258]. However, ERα decreased in kidneys from aged males, but was augmented in those from aged females; ERβ, was not expressed in kidney [222]. The gender effects in the expression of ERs may contribute to sex specific pathology in the elderly [233].

5.4 Progesterone

In 30–49 year old women with normal cycles, progesterone levels are lower than those of younger females at the last stage of the estrus cycle [79]. This decrease seems to be more accentuated during the menopausal transition [79, 223, 228]. Similar to humans, middle-aged female rats experienced a reduction in the levels of progesterone compared to young rats [259]. In contrast, serum progesterone was much higher in aged Long-Evans female rats during the pseudopregnancy phase than in rats experiencing constant estrous or anestrous [107]. Thus, as discussed earlier in the context of estradiol, strain differences must be taken into account when trying to compare the altered rat hypothalamic–pituitary–gonadal axis of aging with that of humans.

Outside of the luteal phase in females, healthy adult men and women do not show significant differences in serum levels of progesterone [187] In two independent studies, no variation in serum progesterone was found with age in males [29, 187]. However, in one study, a progesterone derivative,17-hydroxyprogesterone (which is derived from progesterone via 17-hydroxylase, or from 17-hydroxypregnenolone) was significantly reduced with age [29]. Since most of the 17-hydroxyprogesterone in the male is synthesized in the testis, it has been hypothesized that this decrease probably may reflect a decrease in Leydig cell function [29].

5.5 Progesterone Receptors

The biological effects of progesterone are mediated through the progesterone receptor (PR), which has two isoforms (PR-A and PR-B) [217]. The changes in progesterone levels are associated with age and affect the number, activity, and distribution of PRs. In mammary glands, greater expression of PR was found with advanced age in 30–40-week-old ovariectomized mice in response to estradiol, compared to younger females. Similarly, aged (40-week-old) mice, relative to their younger (10 and 20 weeks old) counterparts, had higher expression of PR [226]. PR expression, on the other hand, was decreased in the rat penis and was linked to erectile dysfunction [225]. There appeared to be no global or marked decline in brain PR with age [37, 45, 78]. Overall, these results indicate that the effects of advanced age on PR expression are determined in a tissue specific manner.

5.6 Testosterone

As a result of abnormalities in the hypothalamic–pituitary–testicular axis during andropause, serum LH and FSH levels increase with age [171]. However, serum LH concentrations often do not parallel the decline in testosterone [171], as a result

of impaired gonadotrophin-releasing hormone secretion and alterations in gonadal steroid feedback mechanisms [245]. In fact, since testosterone is synthesized from estradiol, decreases in total serum levels of testosterone observed in aged men have been explained by a reduction in the circulating levels of free (unbound) estradiol [242]. As noted earlier, some strains of male rodents also experience a decline in serum testosterone. For example, aged CBF1 male mice had decreased levels of testosterone, which was associated with reduced LH, but not FSH, relative to young animals [36]. In contrast, DBA/2J mice showed comparable levels of testosterone at all ages, while C57BL/6J male mice had a very slow rate of decline [61]. Similarly, many aged male rats exhibit a significant decrease in testosterone when compared to younger animals [120, 166, 252].

The mechanisms involved in the age-related decline in serum testosterone of males include primary structural gonadal impairment, degenerative modifications of the pituitary gland, and deficits of the neuro-hypothalamic system. In addition, alteration of peripheral components of the testosterone axis has been found, such as an increase in SHBG and aromatase [109, 241] (which causes the bioconversion of testosterone to estrogens), and a decrease in 5-α-reductase (which converts dihydrotestosterone (DHT) to the active form of testosterone) [241]. Unlike testosterone, DHT cannot be aromatized into estradiol. While these age-related observations are important, it is also crucial to consider the effect of other factors, such as genetics, chronic diseases, medications, obesity, alcohol consumption, smoking, diet, and stress [241].

Irrespective of age, androgens play an important role in women. In fact, female androgen insufficiency can lead to symptoms including fatigue, diminished sense of well-being, decreased libido, and reduction in bone mass, muscle strength, and memory [23]. A decline in total and free testosterone with age has been reported in women [54, 145]. However, reports are inconsistent regarding the levels of SHBG and the effect on testosterone levels in aged women [40, 161]. Using a larger number of subjects, no variation in SHBG with age was reported [54]. However, a more consistent increase in aromatase has been described [109, 110, 204]. The decline in testosterone in women is more pronounced in the early reproductive years, plateaus in midlife, and tends to increase slightly in the later years [54] In contrast, with the sharp decline in estradiol that occurs with menopause an effect on circulating testosterone may not be observed at this time [54].

5.7 Other Androgens

Aside from testosterone, additional androgens, including DHT, androstenedione, DHEA and its sulfated form (DHEAS), may be affected with increasing age. Nevertheless, information regarding age-associated changes of serum DHT in aging men is conflicting. Some have reported increases [70], while others have found no

changes [94, 246] or even decreases in serum levels of DHT [52]. Plasma androstenedione levels decline with age, both in males and in females [94]. Plasma levels of DHEA and DHEAS, secreted mostly by the adrenal glands, are also reduced in both males and females [94, 124, 189]. DHEAS peaked at 20–24 years in men and at 15–19 years in women, then declined steadily in both sexes, though the levels were significantly higher in men than women at ages from 20 to 69 years old [189]. In general, women show a more pronounced androgen decline in their early reproductive years, and a plateau in midlife [54]. Menopause does not produce an abrupt decline in androgens, as it does with estradiol.

5.8 Androgen receptors

The androgen receptor (AR) is a member of the steroid nuclear receptor superfamily that is activated by testosterone and its derivatives [24]. To date, only one AR gene has been identified in humans [158]. AR is mainly expressed in androgen target tissues, such as the prostate, skeletal muscle, the liver, and the central nervous system (CNS). The highest expression levels are observed in the prostate, adrenal gland, and epididymis [124]. It has been reported that aging is accompanied by a decrease in AR concentration in different tissues from men [210] and rodents [31, 205]. In support of the notion that decreased AR is biologically relevant, a CAG-repeat polymorphism of the AR that causes decreased androgen sensitivity has been associated with reduced bone mineral density in men aged 20–50 years [270] and impaired cognitive function in men as they age [261]. The amount of AR declined in the brain cortex of mice of both sexes with advanced age [238]. However, the relative level of AR phosphorylation was significantly higher in aged compared to adult, as well as female relative to male, mice [238]. The significance of differences in the levels of phosphorylation is not clearly understood, but it has been proposed that it might lead to a transformation of AR into a tight nuclear binding form, which is required for downstream hormone activity [238].

6 Changes in Sex Hormones Contribute to Immunosenescence During Normal Aging

6.1 Sex Hormones and the Age-Associated Increase in Circulating IL-6

The well described chronic proinflammatory state in aged individuals without underlying disease [25, 38, 68, 75, 213], is characterized in part by circulating levels of interleukin-6 (IL-6) [65-67]. This age-related increase of IL-6 in serum

begins as early as 30–40 years of age and is more prominent among men [265]. Population studies have identified serum IL-6 levels as a reliable predictor of disability among the elderly [74]. Genetic studies indicated that those who are predisposed to produce low levels of IL-6 during aging—for example, men positive for the polymorphic variant at the 174 C/G locus—appeared to have extended longevity [32]. Moreover, these same investigations indicated that later in life, women experience higher serum IL-6 levels compared to men, in a 174 C/G locus–independent manner [32]. These results suggest that genetics influence longevity in men more than in women. It is possible that environmental factors play a greater role in determining longevity in women or that genetic factors may become prominent later in their life [32].

The increase in circulating IL-6 associated with age can be explained, in part, by the decline in sex hormones, as has been suggested for estrogen, testosterone, and DHEA [231]. In vitro studies using cells obtained from humans and rodents showed that spontaneous increases in the expression and secretion of IL-6 and other proinflammatory cytokines (IL-1 and TNF-α) occurred in macrophages as a result of estrogen deficiency produced by natural [30, 191] or surgical menopause [115, 130, 133, 192, 193], or after discontinuation of estrogen replacement [30, 192]. In vivo cytokine increases, as a consequence of estrogen deficiency, have been more difficult to demonstrate because of technical limitations [196], but similar results to the in vitro observations have been found [42, 87]. In support of these observations, macrophages obtained from ovariectomized mice showed increased expression of components of the IL-6 receptor complex [154]. Since the IL-6 gene lacks the classical estrogen response elements (ERE) in its promoter [203], a mechanism other than direct transcriptional regulation must be present to explain the effects of estrogen on IL-6 production. Perhaps the best described mechanism involves the binding of estrogen-ER/NF-κB dimers to NF-κB binding sites, thereby preventing subsequent transcription [119]. Additionally, exposure to low proestrus levels of estrogen in vivo attenuated the activation of NF-κB in macrophages from young adult mice cultured ex vivo in a model of acute ethanol exposure followed by burn injury [167].

Similar to estrogen deficiency, the age-associated decline in androgens may also upregulate proinflammatory cytokines [196]. Testoterone deficiency induced IL-6 mRNA and protein synthesis in bone marrow cells obtained from young mice after orchidectomy [267]. In vitro, testosterone reduced IL-6 production in macrophages [121], osteoblasts [105], synoviocytes [150] and cell lines [125].

An inverse correlation has also been described for plasma DHEA and circulating IL-6 with age [113, 230]. After in vivo hormone supplementation with DHEA and DHEAS, circulating concentrations of IL-6 [55] and TNF-α were inhibited [131]. The effects of low levels of DHEA on IL-6 production have been observed in splenocytes [113, 128], monocytes [230] and macrophages [132]. However, it has yet to be established whether androgen effects on IL-6, such as testosterone and DHEAS, occur through downstream cell signaling or through indirect mechanisms.

6.2 Sex Hormones and the Age-Related Shift to a Th2 Immune Response

Lymphocytes from aged individuals have decreased proliferation and a decline in production of the Th1 cytokines, IL-2, IFN-γ and IL-12. In contrast, the production of the Th2 cytokines, IL-4, IL-5, IL-6 and IL-10, is increased [81]. These alterations in cytokine secretion produce a shift from a Th1/Th2 balance to a predominantly Th2-phenotype. This, in turn, results in altered immune responses and a higher susceptibility to bacterial and viral infections, as well as to neoplasias [208].

An important contributor to the development of the Th2 phenotype observed in aging is a result of augmented numbers of memory T cells over naive Tcells [169]. When pregnancy and aging were used as variables for different levels of sex hormones, having given birth, parous mice delayed their increase in splenic memory T cells. Also, they augmented the memory/naïve ratio in old mice [27]. In addition, the memory to naïve T cell ratio was lower in aged males [27]. Female mice which have produced offspring exhibited only a slight decrease in circulating IL-2 and an increase in IL-4, IFN-γ, and IL-6 compared with virgin females in association with advanced age [26]. Males, on the other hand, had a smaller decrease in IL-2 during adulthood and lower IFN-γ production with age [26]. From these data, it can be concluded that the onset, magnitude and kinetics of the age-related changes in Th1 and Th2 cytokine production are dependent on the sex hormone status.

6.3 Hormones, Other Major Information Exchange Systems, and Advanced Age

The endocrine, immune, and nervous systems communicate through the release of hormones, cytokines, and neurotransmitters. As aging modifies the functionality of each one of these information exchange systems, it is expected that the interaction between them will also be affected. Straub and collaborators have provided evidence that changes in sex hormones in conjunction with neurotransmitters can contribute to the Th2 shift associated with advanced age [232].

While not a sex hormone, the glucocorticoid cortisol increases the secretion of Th2 cytokines [243, 251] and reduces the production of Th1 cytokines [184, 249, 251]. Similarly, the neurotransmitter, norepinephrine, inhibits the production of Th1 cytokines [62, 71] and augments the levels of Th2 cytokines [62]. However, in contrast with sex hormones, advanced age is associated with a relative increase in cortisol [104, 149] and increased circulating levels of norepinephrine (10–15% per decade over the adult level) [219]. Thus, in addition to sex hormones, imbalances in neurotransmitters can further shift aged individuals towards a Th2 phenotype, perpetuating the defects associated with aging, such as autoimmune

Table 2 Involvement of sex hormones in the manifestation of some aberrant immune responses in advanced age

Condition	References
Normal aging	
Onset of IL-6 increase	[30, 42, 87, 105, 113, 115, 121, 125, 130, 133, 150, 154, 191–193, 196, 203, 230, 231, 267]
Th2 phenotype	[26, 27, 232]
Immune response after injury	
Trauma-hemorrhage	[9, 82, 83, 117, 118, 168]
Burn injury	[1, 90, 127, 138, 140, 155, 200, 240, 139]
Sepsis	[49, 51, 199]
Dermal wound healing	[15, 19–22, 85, 86, 144, 170, 173, 206, 263]

disease, tumor growth, and acceleration of atherosclerosis. The involvement of sex hormones in the manifestation of the aberrant immune responses in advanced age, during normal aging, is summarized in Table 2.

7 Specific Outcomes of Sex Hormone-Related Changes with Age

Independent of age, epidemiologic evidence indicates that sex is a risk factor for trauma and sepsis [reviewed in [7]]. For example, most injury victims are young males [136]. In addition, a higher incidence of bacteremic infections, as well as increased mortality, has been reported in male trauma patients compared with females [33, 162]. The major insults that result in systemic immune dysregulation and are affected by age and sex hormones are hemorrhagic shock, burn injury, and sepsis. After a review on each of these models, the long term effects of aging and sex hormones on wound healing will be discussed.

7.1 Trauma-Hemorrhage

Clinical and experimental studies demonstrated that age and sex are major determinants in the host response following traumatic injury, shock, and/or infection [9, 83, 168]. Following hemorrhagic trauma, female rodents had increased survival and improved cell-mediated immune responses compared to their male counterparts [3, 7, 135, 266]. Additional studies identified testosterone as mainly responsible for the depressed cell-mediated immune responses in males [4-6, 8, 256, 257] and estrogen in enhancement of cell-mediated immune responses in females [6, 8, 77, 134, 135, 262]. Interestingly, some studies showed a reversal of the pattern observed in the young. As opposed to younger injured animals, aged males exhib-

ited enhanced immune responses following injury, when compared to aged females [82, 117]. In addition, macrophages obtained from young males secreted low levels of IL-1β and IL-6 and higher IL-10 than aged subjects Meanwhile, macrophages from aged males released higher levels of IL-1β and IL-6 and reduced IL-10 [117]. In contrast, macrophages isolated from young females following trauma-hemorrhage had enhanced IL-1β and suppressed IL-10 production. Unlike their aged male counterparts, aged females did not have differences in the production of IL-1β and IL-6, but released higher levels of IL-10 secretion [117]. In other studies, splenocyte responses, such as proliferation and the release of IL-2 and IFN-γ, declined in young males but were enhanced in young females after trauma-hemorrhage [118] These effects were reversed in aged animals [118]. Thus, in the trauma-hemorrhage model, the sexually dimorphic cellular response of macrophages and splenocytes in young males and females is reversed, as sex hormone levels decline with age.

7.2 Burn Injury

After burn injury, there is an enhanced systemic inflammatory response, characterized by higher levels of proinflammatory mediators, and defective immune responses, such as DTH and lymphocyte proliferation [59, 141, 269]. Epidemiological studies in burn patients have demonstrated higher mortality in females relative to males sustaining a similar sized burn injury [84, 127, 163, 209]. Similarly, after a 15% total body surface area (TBSA) burn, decreased survival was observed in female mice relative to males [97]. Interestingly, estrogen levels were significantly higher (10–15 fold over baseline) in females following burn injury [58], whereas concentrations of circulating testosterone were decreased [58, 152, 167] These observations suggest that significantly higher levels of estrogen may lead to an improper cell-mediated immune response. Further support for this idea is seen in experiments which show that proestrus levels of estradiol inhibited IL-6, whereas pregnancy levels of the hormone increased the expression of IL-6 [56, 97, 115, 167] Thus, the disparity in the sex-associated outcome between burn injury and hemorrhagic trauma may most likely be due to changes in circulating hormones present after injury.

Aged humans [127] and rodents show higher mortality following burn injury [137] After a 15% TBSA burn, aged mice had a higher mortality rate than young adult mice [137]. In addition, aged female burn–injured mice showed elevated circulating levels of IL-6 and Th2 cytokine production by lymphocytes, but significant decreased DTH response and Con A-stimulated splenocyte proliferation responses compared to young mice [200]. As low, proestrus levels of estrogen suppressed the production of proinflammatory cytokines [47, 95, 234], our laboratory tested the therapeutic efficacy of estrogen supplementation on the immune response following injury in aged female mice. In our studies, low, proestrus levels of estrogen resulted in a marked improvement in survival over a 10-day period

after burn injury [90]. In addition, attenuated serum levels of IL-6 were observed, in conjunction with a partial restoration of the DTH response [140]. A recovery in IFN-γ, but not in IL-4 production, suggested a restoration of the Th1-Th2 shift, as a result of the estrogen treatment [139]. Overall, our results demonstrate that using the immunomodulatory properties of estradiol has beneficial effects in aged, injured subjects.

7.3 Sepsis

Retrospective studies indicate that men have increased morbidity and mortality from sepsis as compared to women [9, 33, 160, 215, 266]. After sepsis-induced cecal ligation and puncture in rodents, splenocytes from septic males exhibited reduced proliferative capacity and decreased production of IL-2 and IL-3, but not in those from female septic mice [266]. In similar studies, higher plasma levels of IL-1β were found in female mice versus male mice after LPS administration, as a model of the inflammatory response provoked during infection [153]. These data suggest that better cellular responses and higher levels of proinflammatory cytokines may contribute to the improved response in females relative to males during sepsis.

Infectious diseases comprise one of the ten major causes of death in the elderly [264]. Moreover, pneumonia, influenza, and complications of bacteremia in this age group are associated with a poor prognosis. Elderly patients hospitalized with *Streptoccocus pneumoniae* infection, had prolonged elevation of circulating proinflammatory cytokines [39], as do aged volunteers given endotoxin [142]. In animal models, aged mice given LPS were approximately six times more sensitive to the lethal toxicity than young mice [237]. In addition, LPS exposure induced higher serum and tissue levels of IL-6, IL-1β and TNF-α in aged mice as compared to young [88, 89, 169, 237].

The effect of age and sex on cytokine production has also been studied in peripheral blood mononuclear cells isolated from young and elderly subjects [199]. After in vitro stimulation with LPS, decreased intracellular levels of TNF-α and IL-6 were detected in monocytes from elderly women, relative to young women [199]. In contrast, monocytes from elderly males showed an elevated number of cells positive for both IL-1β and TNF-α after LPS stimulation [199]. In different analyses, spontaneous production of the chemokine IL-8, was decreased in macrophages obtained from elderly males, as compared to that of aged females [49]. Upon in vitro stimulation with LPS, production of IL-8 by macrophages from elderly males was increased over levels in young males. However, cells from aged females showed no change compared to cells from young donors [49]. The involvement of sex hormones in the response to LPS with aging was further supported by the observation that castrated young male rats exhibited similar macrophage function to aged male rats [51].

7.4 Wound healing

The process of wound healing can be separated into the following overlapping phases: hemostasis, inflammation, proliferation, and resolution [57]. These four phases have been studied in detail and exhibit impairment in association with aging [reviewed in [91]]. The detrimental effects of aging on the healing of acute wounds include a prolonged inflammatory response [18, 235], upregulation of protease activity [17], and reduced extracellular matrix deposition [16].

Elderly men heal more slowly than do elderly women. Interestingly, this is true even when both sexes receive estrogen treatments [19]. Estrogen treatment has been shown to accelerate the rate of acute healing in men, and particularly in elderly women, by reducing the inflammatory response [15]. On the contrary, testosterone significantly delayed acute healing in aged humans, as a result of an increased inflammatory response [21]. This suggests that, besides the alterations in immune status [168, 220], the age-associated decrease in sex hormones [86] may also contribute to sex differences in wound healing in the elderly.

The salutary effect of estrogen in wound healing includes lessened inflammatory cell infiltration [19, 170] and inhibition of the proinflammatory cytokines macrophage migration inhibitory factor (MIF) and TNF-α [22]. In addition, estrogen improved the rate of re-epithelialization [20], promoted angiogenesis [173], and stimulated wound contraction [206].

In elderly men, elevated serum testosterone levels correlated with delayed healing of excisional punch wounds [21]. The use of animal models has allowed us to gain a great deal of information regarding the mechanisms involved in this phenomenon. In mice, systemic administration with the AR antagonist, flutamide, improved wound repair, decreased DNA-binding activity of NF-κB, and lowered the production of TNF-α. In other studies, macrophages isolated from the wound site directly upregulated the proinflammatory cytokines, TNF-α [21] and IL-6 [85] in response to testosterone. This evidence suggests a possible role for the AR in impaired healing and increased wound inflammation [21]. However, careful interpretation of these results is required, as there is conflicting evidence showing both inhibitory and stimulatory effects of testosterone in the production of proinflammatory cytokines in vitro [86]. The participation of sex hormones in the manifestation of the aberrant immune responses in advanced age after injury is summarized in Table 2.

8 Therapeutic Benefits Versus Detrimental Effects of Hormone Replacement Therapies

As discussed in the previous pages, experimental research and observational clinical data have provided evidence for the beneficial effects of hormone replacement therapy (HRT) on the aging process. However, large trials have recently called into

question whether the effects of some forms of HRT are truly advantageous, potential hazards and concerns have even been raised. In this section, we will briefly discuss the results of recent HRT trials.

8.1 Estrogen and Progesterone

As noted above there is a vast array of immunomodulatory properties of estrogen and progesterone [217]. Thus, the accumulation of basic and clinical data prompted the development of interventional studies to analyze the therapeutic effects of HRT. Postmenopausal estrogen therapy, alone and in combination with progestin, involves approximately 100 years of research and 75 years of clinical practice [254]. However, for the last few years, evidence has surfaced against the beneficial effects of estrogen replacement [129].

The Heart and Estrogen/Progestin Replacement Study (HERS) compared the effects of conjugated equine estrogens plus medroxyprogesterone acetate on cardiovascular function in 2,763 women with prior coronary disease. The results showed an increase in coronary heart disease in women taking HRT [108]. The Women's Estrogen for Stroke Trial (WEST), a randomized, double-blind, placebo-controlled trial, assessed the effects of estradiol therapy in 664 postmenopausal women (mean age, 71 years) who already had an ischemic stroke or transient ischemic attack. In this study, no benefit of estrogen treatment on cerebral stroke incidence was found and, in fact, an increased risk of fatal stroke was reported [250]. The Women's Health Initiative (WHI), a large, placebo-controlled trial involving more than 16,000 women aimed to study the effects of estrogen therapy alone or combined estrogen plus progestin. Benefits included decreased risks for colorectal cancer, beginning at 3 years, and for hip fracture over time [212]. This study was terminated in 2004 resulting from findings of an increased risk of breast cancer, cardiovascular complications, ischemic stroke, levels of inflammatory biomarkers, and dementia, including Alzheimer's disease (reviewed in [217]). Other studies have indicated that the greatest benefits of estrogen replacement are increased bone density and decreased risks of fractures [129]. Subsequent to these trials, many medical organizations recommend that estrogens should not be used in women over the age of 60 years [217]. Accordingly, the use of estrogen currently is recommended only temporarily for women who undergo surgical or natural premature menopause [129] and for short-term control of hot flashes at the beginning of menopause [69].

As an aftermath of the HERS, WEWT and WHI trials, the recommended dosages of estrogen and estrogen–progestin therapies have markedly decreased since they first were introduced. Data demonstrating that benefits in vasomotor and vulvovaginal symptoms, prevention of bone loss, and protection of the endometrium in association with aging can still be achieved with lower doses than the commonly prescribed ones [254].

8.2 Testosterone

Most of the information regarding the benefits of testosterone replacement therapy has been postulated from studies involving younger hypogonadal patients and animal models [236]. In younger men, the benefit to risk ratio is high. However in aged males, potential risks have not been assessed, so the benefit to risk ratio of testosterone replacement therapy in the aging male is still not known [124].

In older, hypogonadal males, continued testosterone therapy increases muscle mass [124, 129]. In one study, transdermal testosterone was administered to 123 subjects continuously for up to 42 months [253]. Continuous treatment normalized testosterone levels, increased the mean serum estradiol to testosterone ratio, and suppressed mean serum FSH and LH levels [253]. In addition, lean body mass augmented as early as 3 months, while fat mass decreased. These changes were maintained with treatment, but were not accompanied by significant increments in muscle strength [253]. As a caveat, however, this study was neither placebo-controlled nor powered to determine the effects of the treatment on prostate cancer risk.

Testosterone treatment has been shown to improve libido in both males and females [129]. However, information from controlled trials, specifically on sexual function in the elderly, remains scarce [28, 124]. In some small studies, parameters of sexual function in elderly men have been shown to be improved compared with placebo treatment [143, 179]. Nevertheless, a lack of an effect has been reported in trials using the anabolic androgen, oxandrolone [216], an aromatase inhibitor [151], or DHT [143]. Additional studies are needed to accurately make conclusions on the effects of androgen administration on sexual function in elderly men. In many short term studies, testosterone therapy has shown an improved, but rather modest, sexual function in elderly women compared with those on estrogen or placebo [reviewed in [28]]. However, secondary effects, such as supraphysiological and unpredictable levels of testosterone to an adverse lipid profile, have been observed. Thus, androgen replacement to improve sexual function in aged females may not be the best choice of therapy [28].

The effects of testosterone on cardiovascular disease include improvement of cardiovascular efficiency [63], reduced incidence of angina [63], and improved cardiac muscle remodeling and coronary artery vasodilation [122, 126]. However, when the effects of androgen therapy on cardiovascular risks have been analyzed, the findings have generally been unremarkable. This dampens the potential of using androgen therapy to prevent the occurrence or to improve the outcome of cardiovascular diseases in elderly men [124]. In addition, side effects can be seen with long term testosterone treatment using near physiologic doses. Polycythemia is the major side effect [129], but long term administration of testosterone or DHT may also increase the risk of prostatic carcinoma and benign prostatic hyperplasia [176]. Other side effects include sleep apnea, breast development in men, breast carcinoma, fluid retention, hypertension, alterations in the lipid profile, and atherosclerosis [101, 207].

8.3 DHEA

A great deal of information has been accumulated in recent years regarding the beneficial effects of pharmacological doses of DHEA. Potential benefits include cardioprotection, antiobesity, immunostimulation, and neuroprotection [129]. The effect of DHEA on the improvement of immune function in the elderly was evidenced by observations in aged mice showing that immunization shortly after an oral dose of DHEAS provided adjuvant effects that improve immunity against influenza [11, 12]. However, in a prospective randomized, double-blind study, in which participants received either DHEA for 4 consecutive days starting 2 days before immunization or placebo, there was no improvement in the age-related decrease response to immunization against influenza [53]. In conclusion, more studies are necessary to justify the use of DHEAS as an adjuvant for the elderly.

9 Conclusions

Analysis of the contribution of sex hormones to different aspects of imnunosenescence has been presented in this chapter. After a review of the enormous amount of literature on the field, one may conclude that changes in the sex hormone environment can contribute to immunosenescence. Thus, sex hormone status can help shape normal aspects of the aging immune system, as well as immune responses to injury and infection. Additionally, we can conclude that immunosenescence is a manifestation of the continuous interplay between the immune, the endocrine, and the nervous systems over time. Overall, when trying to determine the best treatment option for any number of pathological conditions, it is important to consider both age and sex as potential modifiers of the disease process. Thus, therapies targeted to the specific hormonal and immune status of an individual may prove to be of most benefit for optimal clinical outcomes.

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Glucocorticoids and DHEA: Do They Have a Role in Immunosenescence?

Moisés E. Bauer, Cristina M. Moriguchi Jeckel, Cristina Bonorino, Flávia Ribeiro and Clarice Luz

Contents

Abstract: This chapter summarizes recent work suggesting that human immunosenescence may be closely related to both psychological distress and stress hormones. The age-related immunological changes are also similarly found during chronic

M. E. Bauer (✉) · C. Bonorino
Faculdade de Biociências
Instituto de Pesquisas Biomédicas
Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS)
Av. Ipiranga 6690, 2º andar
P.O. Box 1429. Porto Alegre, RS 90.610-000, Brazil
E-mail: mebauer@pucrs.br

C. M. Moriguchi Jeckel
Faculdade de Farmácia,
PUCRS, Av. Ipiranga, 6681.
Porto Alegre, RS 90619-900, Brazil

F. Ribeiro
Ageing and Tumour Immunology Group,
University of Tübingen,
Sektion Transplantionsimmunologie / Immunhämatologie,
Waldhörnle Strasse 22, D- 72072 Tübingen, Germany

C. Luz
LabVitrus, Rua Garibaldi,
659/502, Porto Alegre,
RS 90035-050, Brazil

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stress or glucocorticoid exposure. It follows that endogenous glucocorticoids (cortisol) could be associated to immunosenescence. When compared with young subjects, healthy elders are emotionally distressed in parallel to increased cortisol/dehydroepiandrosterone (DHEA) ratio. Furthermore, chronic stressed elderly subjects may be particularly at risk of stress-related pathology because of further alterations in glucocorticoid-immune signaling. Although DHEA and its metabolites have been described with immune-enhancing properties, their potential use as hormonal boosters of immunity should be interpreted with caution. The psychoneuroendocrine hypothesis of immunosenescence is presented in which the age-related increase in the cortisol/DHEA ratio is major determinant of immunological changes observed during aging. We finally discuss that strictly healthy elders are largely protected from chronic stress exposure and show normal cortisol levels and T-lymphocyte function. This information adds a new key dimension on the biology of aging and stress.

Keywords: Aging • Immunosenescence • Glucocorticoids • Lymphocytes

1 Introduction

Aging is a continuous and slow process that compromises the normal functioning of various organs and systems in both qualitative and quantitative terms. The clinical consequences of immunosenescence may include increased susceptibility to infectious diseases, neoplasias and autoimmune disease (Castle 2000). This altered morbidity is not evenly distributed and should be influenced by other immune-modulating factors, including genetic background and chronic stress exposure (Bauer 2005). Indeed, several immunosenescence-related changes (e.g., thymic involution, lower counts of naïve T-cells and blunted T-cell proliferation) resemble those observed following chronic stress (McEwen et al. 1997; Selye 1936) or glucocorticoid (GC) treatment (Fauci 1975).

In addition to immunosenescence, the endocrine system also undergoes important changes during aging (endocrinosenescence). It has been demonstrated a decline in growth hormone (GH), sex hormones and dehydroepiandrosterone (DHEA) with aging (Roshan et al. 1999). DHEA is the major secretory product of the human adrenal and is synthesized from cholesterol stores (Fig. 1). The hormone is uniquely sulphated (DHEAS) before entering the plasma, and this prohormone is converted to DHEA and its metabolites in various peripheral tissues (Canning et al. 2000). Following secretion, total DHEA in the circulation consists mainly of DHEAS—the serum concentration of free DHEA is less than 1%. Serum DHEA levels decrease by the second decade of life reaching about 5% of the original level in the elderly (Migeon et al. 1957). It has been suggested that DHEAS/DHEA may antagonize many physiological changes of endogenous glucocorticoids (Hechter et al. 1997) including enhancing immunomodulatory properties.

There is also evidence suggesting that aging is associated with significant activation of the hypothalamic-pituitary-adrenal (HPA) axis (Halbreich et al. 1984; Heuser

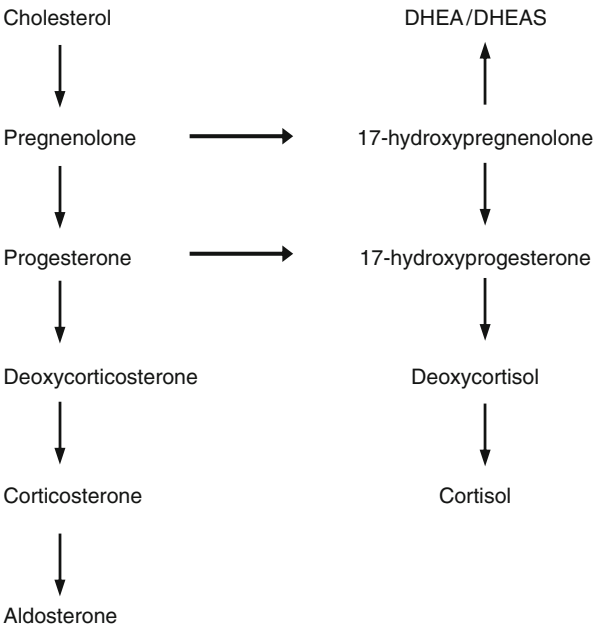

Fig. 1 Adrenal steroidogenic pathways.

et al. 1998; Luz et al. 2003) in increased production of cortisol in the man. The HPA axis is pivotal for the homeostasis of the immune system and its dysregulation has been associated with several immune-mediated diseases. For instance, HPA axis over-activation, as occurs during chronic stress, can affect susceptibility to or severity of infectious disease through the immunosuppressive effect of the glucocorticoids (Kiecolt-Glaser et al. 1996); (Vedhara et al. 1999). In contrast, blunted HPA axis responses are associated with enhanced susceptibility to autoimmune inflammatory disease (Sternberg 2002). It is noteworthy to mention that elderly subjects are particularly at risk for both infectious and chronic inflammatory diseases. Furthermore, chronic inflammatory diseases may be associated with premature aging of the immune system and present several similarities of immunosenescence including shortening of cellular telomeres, decreased T-cell receptor specificities, loss of naïve T-cells and increased production of proinflammatory cytokines (Straub et al. 2003). Dysregulation of the HPA axis may contribute to but it is not solely responsible to immunosenescence. Chronic stressed elderly subjects may be at risk of stress-related pathology because of further alterations in GC immunoregulation (immune signaling).

The present chapter summarizes recent findings that suggest that immunosenescence may be closely related to both psychological distress and stress hormones. In particular, striking similarities of immunological changes are found during aging, stress exposure or GC treatment in vivo. The neuroendocrine hypothesis of immunosenescence is reconsidered in which both the psychological distress and increased cortisol/DHEA (C/D) ratio are thought to be major determinants of immunological changes observed during aging. We also discuss the protective effects of a strictly health status during chronic stress exposure during aging.

2 The SENIEUR Protocol

It remains controversial whether immunosenescence cause or are caused by underlying disease commonly observed in elderly populations. Therefore, strenuous efforts have been made to circumvent this problem by separating "disease" from "aging", as exemplified by the application of the SENIEUR protocol (Ligthart et al. 1984) that defines rigorous criteria for selecting healthy individuals in immunogerontological studies. The health conditions are checked accordingly to clinical investigations and to hematological and various biochemical parameters. The exclusion criteria includes: infections, acute or chronic inflammation, autoimmune diseases, heart disease, undernourishment, anemia, leucopenia, clinical depression, neurodegenerative disease, neoplasia and use of hormones and drugs. Based on this protocol, it is possible to select up to 10% of strictly healthy volunteers from elderly populations.

3 Healthy Aging is Associated with Emotional Distress and Increased Cortisol/DHEA Ratio

Psychological distress may be an important risk factor for immunosenescence. Human aging has been associated with several psychological and behavioral changes, including difficulty to concentrate, progressive cognitive impairments and sleep disturbances (Howieson et al. 2003; Piani et al. 2004). Although individually identified, these alterations may be associated with major depression. Indeed, depression is highly prevalent in several age-related chronic degenerative diseases, including cardiovascular diseases, Parkinson's disease, Alzheimer's dementia, cancer and rheumatoid arthritis (Dew et al. 1998). In addition, both aging (Gabriel et al. 2002) and major depression (Schiepers et al. 2005; Trzonkowski et al. 2004) have been associated to increased levels of proinflammatory cytokines and could thus contribute for further immunological diseases in the frail elderly.

We have recently demonstrated that healthy aging was associated with significant psychological distress. In particular, it was found that SENIEUR elders were significantly more stressed, anxious and depressed than young adults (Collaziol et al. 2004; Luz et al. 2003). Several stressors were ascribed to the healthy elders, including: feeling unable to work or having problems to perform their house work, sexual problems and reduced libido, loss of a relative or friend, and social exclusion. The literature regarding age-related psychological changes is controversial and others did not find these changes (Nolen-Hoeksema and Ahrens 2002). This could be due to methodological issues, since specific clinical interviews are required to assess depression in the elderly.

In parallel to psychological distress, we have also observed that SENIEUR elders had significantly higher (~45%) salivary cortisol production throughout the day compared to young adults (Luz et al. 2003). Cortisol peaked in the morning and presented a nadir at night, with a regular circadian pattern for both groups. These

data further suggest that healthy aging is associated with significant activation of the HPA axis (Deuschle et al. 1997; Ferrari et al. 2000; Ferrari et al. 2004; Halbreich et al. 1984; Heuser et al. 1998; Van Cauter et al. 1996). However, some previous studies have also observed an flattened diurnal amplitude of ACTH and cortisol levels during aging (Deuschle et al. 1997; Ferrari et al. 2004). Increased cortisol levels are also seen in demented patients (Maeda et al. 1991), major depression (Gold et al. 1988) or during chronic stress (Bauer et al. 2000; Kirschbaum et al. 1995).

In addition, it was observed that healthy elders had lower DHEA levels (-54%) throughout the day compared to young adults (Luz et al. 2006). Furthermore, elders also displayed a flat circadian pattern for DHEA secretion. The morphological correlates of the age-related changes of DHEAS/DHEA secretion are progressive atrophy of the zona reticularis of adrenal glands (Ferrari et al. 2001). The lack of appropriate DHEA levels could be another detrimental factor during immunosenescence since this hormone has immune enhancing properties (as further discussed in this chapter).

The higher cortisol in parallel to lower DHEA levels will consequently lead to higher C/D ratios throughout the day. The assessment of molar concentrations constitute another way to evaluate the adrenal function in the organism (Butcher and Lord 2004; Ferrari et al. 2001; Straub et al. 2000). The measurement of isolated hormonal samples may be an oversimplification and the C/D ratio may contribute to the effective determination of functional hypercortisolemia. The impaired DHEA secretion, together with the increase of cortisol, results in an enhanced exposure of various bodily systems (including brain and immune system) to the cytotoxic and modulatory effects of GCs. Some brain cells (hypocampus) and lymphocytes are specially targeted by the cortisol because they express higher densities of mineralo receptors (MRs) and GC receptors (GRs) (McEwen et al. 1997). The peripheral tissues of elders may be thus more vulnerable to the GC actions in a milieu of low protective DHEA levels. The antagonist action of DHEA to cortisol in the brain suggests that measurement of cortisol alone may provide an incomplete estimate of hypercortisolemia.

In our previous study, psychological distress was positively related to salivary cortisol levels and negatively correlated to DHEA levels during aging (Luz et al. 2003). Therefore, it becomes difficult to dissociate these neuroendocrine changes observed in the elderly with those produced by psychological stimuli. It should be also pointed out that endocrinosenescence includes a substantial decline in several hormones, including growth hormone, testosterone, progesterone and aldosterone—all of which with reported immunomodulatory properties. Thus the endocrinosenescence may be considered as another risk factor for immunosenescence.

3.1 The Glucocorticoid Cascade Hypothesis

Cumulative neural damage produced by stressors during life may contribute to increased HPA function during aging. In this context, peripheral GCs may have an important role in damaging key brain areas involved with regulation of the HPA

axis. Evidence for GC involvement in hippocampal aging led to the establishment of the "glucocorticoid cascade hypothesis" (Sapolsky et al. 1986). This hypothesis states that GCs participate in a fed-forward cascade of effects on the brain and body. In this case, progressive damage to the hippocampus, induced by GCs, promotes a progressive elevation of adrenal steroids (i.e. cortisol) and dysregulation (down-regulation of GC receptors) of the HPA axis (Sapolsky et al. 1986). The glucocorticoid cascade hypothesis of aging is a prime example of "allostatic load" (McEwen 1998; McEwen 2003) since it recognizes a mechanism that gradually wears down a key brain structure, the hippocampus, while the gradually dysregulated HPA axis promotes pathophysiology in tissues and organs throughout out the body. The net results of the age-related hippocampal damage are impairment of episodic, declarative, spatial, and contextual memory and also in regulation of autonomic, neuroendocrine, and immune responses. It should be mentioned that the effects of glucocorticoids on the hippocampus are reversible.

Sapolsky and col. (1986) have also proposed that several age-related pathologies are also observed following excessive glucocorticoid exposure and include muscle atrophy (Salehian and Kejriwal 1999), osteoporosis/hypercalcemia (Tamura et al. 2004), hyperglycemia/hyperlipidemia, atherosclerosis, type II diabetes and major depression (Juruena et al. 2003; Lee et al. 2002).

4 Similarities between Aging and Chronic Glucocorticoid Exposure

We have now discussed that healthy aging is associated with psychological distress in parallel to increased C/D ratio. All leucocytes exhibit receptors for the neuroendocrine products of the HPA and sympathetic-adrenal medullary axes. It seems reasonable to speculate that increased cortisol and lower DHEA may thus contribute to immunological changes observed during aging. This section will provide significant evidence that the immunological changes observed during aging are also similarly found during psychological stress or chronic GC exposure.

4.1 Changes in Cellular Trafficking

Trafficking or redistribution of peripheral immune cells in the body is of pivotal importance for effective cell-mediated immune responses. Aging is associated with several peripheral enumerative changes in leukocytes, including a decrease of naive (CD45RA+) and an increase of memory (CD45RO+) T-cells, an expansion of CD28- T-cells or an increase of natural killer (NK) cells (Gabriel et al. 1993; Globerson and Effros 2000; Hannet et al. 1992; Martinez-Taboada et al. 2002). Overall, cellular components of the innate immune system (e.g., monocytes, neutrophils and NK-cells) seems to be preserved during aging in contrast to several

age-related decrements in adaptive immune responses—especially T-cells (Pawelec et al. 2002). However, T-cells are also especially targeted in the same direction during chronic stress exposure (Biondi 2001) or following GC treatment in vivo(Bauer et al. 2002; McEwen et al. 1997) (see Table1). Immunologists have recently characterized a new T-cell subset (CD4+C25+FoxP3+) with important regulatory role in suppressing excessive or misguided immune responses that can be harmful the host. These lymphocytes were called regulatory T (Treg) cells and are responsible for turning off immune responses against self antigens in autoimmune disease, allergy or commensal microbes in certain inflammatory diseases (Fontenot et al. 2003; Sakaguchi 2000). It was interestingly found that aging, glucocorticoid or chronic-stress can increase peripheral Treg cell numbers (Hoglund et al. 2006; Navarro et al. 2006; Trzonkowski et al. 2006). In spite of the several similarities among age- and stress-related immunological alterations, only a few studies have addressed the role of stress factors on human immunosenescence.

We have recently investigated the role of psychoneuroendocrine factors in regulating the distribution of peripheral T-cell subsets during healthy aging (Collaziol et al. 2004). The mechanisms underlying the regulation of the peripheral pool of lymphocytes are still largely unknown. It has been speculated that CD95 (APO1/Fas) may be involved in this process through engagement of apoptosis (Potestio et al. 1999). CD95 is a member of tumour necrosis factor (TNF) family and its ligand (CD95L) is found on activated T-cells (Nagata and Golstein 1995). The CD95-CD95L binding seems to play an important role in maintaining the cellular homeostasis of the immune system and may contribute to stress-related changes in cell trafficking (Yin et al. 2000). Confirming previous reports, we recently demonstrated that changes in lymphocyte distribution were noted in the elderly as demonstrated by a significant drop in naïve T-cells associated with higher expression

Table 1 Changes in cellular trafficking. Direction of arrows indicate increase (⇧), decrease (⇩) or no change (⇔) compared to corresponding control levels. ? = data not available; NK, natural killer; Treg = T-regulatory; CD3+CD45RA+, naïve T-cells; CD3+CD45RO+, memory T-cells. Based on references (Bauer et al. 2002; Biondi 2001; Fauci 1975; Globerson and Effros 2000; Hoglund et al. 2006; McEwen et al. 1997; Navarro et al. 2006; Trzonkowski et al. 2006)

Cell	Aging	Stress	GC treatment
Neutrophils	⇔	⇧	⇧
Monocytes	⇔	⇩	⇔
NK cells	⇧	⇧	⇧
B cells	⇩	⇩	⇩
CD4+ T cells	⇩	⇩	⇩
CD8+ T cells	⇩	⇩ or ⇧	⇩
Treg cells	⇧	⇧	⇧
CD3+CD45RA+	⇩	⇩	⇩
CD3+CD45RO+	⇧	⇩ or ⇧	⇩
CD3+CD28-	⇧	?	?

of CD95 in this subset (Collaziol et al. 2004). We have speculated this differential expression of CD95 may potentially select naive T-cells for apoptosis and could further explain age-related reductions in CD45RA+ (naïve) cells. Furthermore, healthy elders were significantly distressed and stress scores were found positively associated to CD95 expression on CD45RA+ cells.

Glucocorticoids may also contribute to the numerical cellular changes observed during aging. It has been demonstrated that GC-induced apoptosis on monocytes is at least partially mediated by the expression of both CD95 and CD95L (Schmidt et al. 2001). Another study showed that glucocorticoids may either induce T-cell apoptosis in a CD95-independent manner, or protect T-cells from CD95-mediated apoptosis (Zipp et al. 2000). Furthermore, there is some evidence that psychological stress may regulate the proportion of peripheral lymphocytes via the expression of CD95. It has been demonstrated that chronic stress may induce lymphocyte apoptosis in mice (Yin et al. 2000) or in man (Oka et al. 1996) via upregulation of CD95. Our results support the concept that age- or stress-related increase in cortisol levels may be preferentially altering the expression of CD95 on CD45RA+ cells. Preliminary data from our laboratory indicate that human CD45RA+CD95+ cells are in fact more sensitive to dexamethasone (DEX) treatment in vitro(unpublished results). There is some data suggesting that human naïve T CD4+ cells are more sensitive to DEX than memory T CD4+ cells (Nijhuis et al. 1995). Overall, our results suggest that there are complex psychoneuroendocrine interactions involved with the regulation of the peripheral pool of lymphocytes. In particular, it was shown that both psychological stress and GCs synergize during aging to produce alterations in T-cell trafficking.

4.2 Changes in Innate Immunity—Focus on DCs

To date, the effects of stress or aging on dendritic cells (DC) are largely unknown. These professional antigen presenting cells play a determinant role on the interface between innate and adaptive immunity (Steinman 2003). They sense pathogens through a myriad of toll-like receptors, endocytose them and produce immune mediators that lead to inflammation, such as TNF-α and nitric oxide (NO). They also secrete cytokines that are key to the development of specific, adaptive responses, such as type I interferons (IFN-α and $-\beta$), and IL-12. DC process antigens from pathogens and present them to T-cells and the concentration of antigen, the magnitude of co-stimulatory signals such as CD86 delivered, together with the cytokines produced, set up the stage for T-cell responses. Antigen presentation by mature DC leads to the initiation of immune responses, and the predominant cytokines produced can skew the response towards a TH1 or TH2 phenotype (Banchereau et al. 2000). Presentation by immature DC, however, can result in tolerance, in some situations even leading to the recruitment of regulatory, CD4+CD25+Foxp3+ T-cells (Luo et al. 2007; Yamazaki et al. 2006). Thus, DC play a fundamental regulatory role in immunity.

An intriguing aspect of dendritic cell biology is the existence of different subpopulations (Vremec and Shortman 1997). These can be distinguished by the expression of different surface markers, are distributed in different body compartments, and possess different functions. Also, some are derived from distinct precursors. Basically, both in human and mice, two subpopulations can be identified; tissue derived, and blood derived cells. Tissue derived DC include Langerhans cells (LC) and interstitial cells, that respectively reside in skin and tissues. They capture antigen in the periphery and migrate to lymph nodes, to interact with other DC and T-cells. Blood derived DC are replenished in lymphoid organs from the blood, and are generally designated as plasmacytoid (important for anti-viral immunity-(Banchereau et al. 2000), myeloid or lymphoid, these latter ones apparently responsible for cross-presentation (the ability to present endocytosed antigens in MHC Class I molecules—(Brossart and Bevan 1997). DC can be derived in vitro directly from bone marrow precursors (Inaba et al. 1992) and also from circulating monocytes, although the cells that arise from these cultures do not directly correspond to the same populations identified in vivo. Because so little is known about antigen presentation and T-cell activation by each subpopulation, it is important to identify the effects of stress on different DC populations, as well as how that relates to immunosenescence.

Probably, the most studied effect of chronic stress over dendritic cell function is the modulatory function of glucocorticoids. For example, in vitro studies show that murine bone marrow differentiated DC treated with DEX show downregulation of the costimulatory molecules CD86, CD40, CD54, as well as of MHC Class II, but not MHC Class I, molecules (Pan et al. 2001). This study also verified a decreased capacity of MHC class II presentation of antigens, but not of endocytic activity for DEX treated DC, and a reduction on their production of interleukin (IL)-1β and IL-12. It has also been reported that glucocorticoids can downregulate the production of TNF-α and IL-12, but not IL-10, by DC, and thus are able to affect skew T-cell responses towards a TH2 phenotype (Elenkov et al. 2000). Studies with glucocorticoids applied to skin in vivo for 7 days showed a reduction in the number of LC in situ, as well as a reduction in expression of costimulatory molecules, leading to reduced alloreactive stimulatory capacity (Ashworth et al. 1988). Accordingly, in transplant models, DEX has been shown to affect differentiation and reduce costimulatory function of DC (Abe and Thomson 2003) suppressing MHC Class II and CD86 expression (Muller et al. 2002), and consequently DC maturation in vitro. In the same study, treatment with DEX during graft procedure reduced DC, as well as T-cell, infiltration on the graft.

There are very few studies on DC function in experimental systems of psychological stress. One study found that the increase in corticosterone levels correlated with decreased processing of viral antigens and their presentation in MHC Class I molecules, leading to decreased antiviral immune responses (Truckenmiller et al. 2005). Their results pointed to an effect over the processing machinery of all cells, suggesting stress can profoundly affect the protein processing pathways. Finally, glucocorticoids can induce natural anti-inflammatory cells through DC. Studies with bone marrow derived DCs showed that glucocorticoids can not only impair development of immature DC into mature DC, but also that multiple restimulation

of CD4+ T-cells with DEX treated DC can lead to the expansion of T-cells with the regulatory phenotype (CD4+CD25+) (Matyszak et al. 2000), which are vital for the control of inflammation and autoimmunity in vivo.

Curiously, DHEA and DEX appear to have somewhat opposing effects over the differentiation of dendritic cells from monocyte precursors. The only study comparing the 2 hormones (Canning et al. 2000) showed that continuous presence of DHEA on dendritic cell cultures from monocytes in the presence of GM-CSF and IL-4 leads to the accumulation of immature DC, although markers like CD80 or CD40 are only slightly altered compared to the control. Cultures of monocytes in the same conditions, only continuously supplemented with DEX, however, leads to their differentiation into macrophage-like cells, with high CD14 expression, and low surface CD80 and CD40, with almost no IL-12, but high IL-10 production.

Aging has been associated with similar changes in DC function. While some report no changes in surface expression of MHC Class II and CD86 in aged in vitro monocyte-derived DC (Agrawal et al. 2007b) or in vivo DC (Lung et al. 2000), others have observed a markedly reduced expression of HLA-DR (Pietschmann et al. 2000) for monocyte enriched, lymphocyte depleted peripheral blood cells of aged subjects. Also, the numbers of LC in gingival epithelium (Zavala and Cavicchia 2006) or and skin (Bhushan et al. 2002) appear to be diminished in aged individuals. A normal TNF-α and IL-12 production by monocytes-derived DC from aged subjects was reported (Lung et al. 2000), but an increased TNF-α and IL-6 response to LPS was found by others (Agrawal et al. 2007a), as well as a decreased migratory and phagocytic capacity. Monocyte-derived DC from elderly individuals were not impaired in their ability to induce T-cell responses (Grewe 2001) or proliferation of T-cell lines (Steger et al. 1997). However, the efficacy of autologous DC-based antitumor vaccines was impaired in aged individuals (Sharma et al. 2006).

In our laboratory, we compared the effects of stress induced glucocorticoids and aging on the differentiation of bone marrow derived DCs. Results are shown in Fig. 2. After seven days of culture with IL-4 and GM-CSF (Inaba et al. 1992), murine bone marrow cells consistently yield three distinct populations of DC, as determined by MHC Class II and CD86 expression (Fig. 2). The population in the upper right quadrant of the plots (Class II hi, CD86 hi) represents the mature DC, while the population in the middle (Class II lo, CD86 lo) contains the immature DC. The population in the bottom left quadrant is negative for both markers and has not yet started to differentiate. Bone marrow cultures from 6 month old mice yielded precisely these populations (in A and C). However, treatments with 10^{-7} M DEX (B) on day 5 of culture lead to an arrest of dendritic cell differentiation, leaving the cells mostly at the immature stage. A similar pattern was observed in D, when bone marrow of a 2 year old mouse was cultured in the same conditions as A and C. Consequently, aged bone marrow produced mostly immature DC.

Together, these results consistently point to an inhibitory effect of stress, aging and glucocorticoids over DC function. They also suggest that these GCs can affect immunoregulation, modulating the TH1/TH2 decision and also leading to the generation of regulatory T-cells. These are pleiotropic effects, and it is likely that a variety of mechanisms is involved.

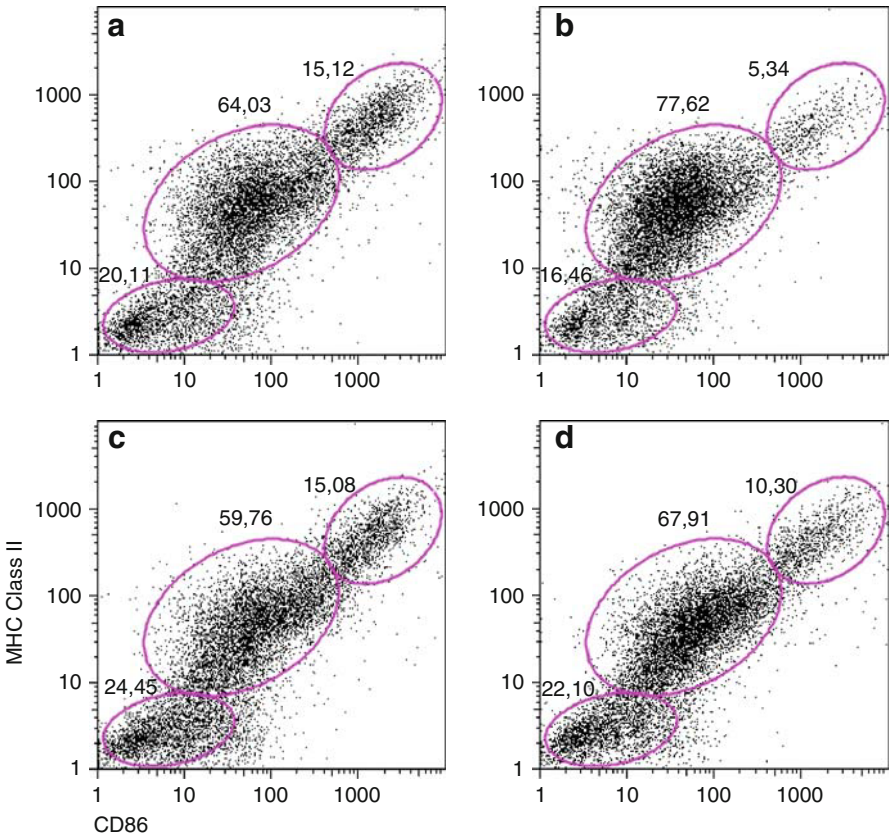

Fig. 2 Dexamethasone and aging lead to an arrest in the maturation phenotype of DC. Bone marrow cells from young (6 months: A, B and C) or aged (2 year old:D) mice were cultured in vitro with IL-4 and GM-CSF. In C, 10^{-7}M dexamethasone was added to the cultures on day 5. Numbers represent the percentages of gated populations.

4.3 Changes in Cell-mediated Immunity

Although many components of the immune system show age-related changes, T-cells show most consistent and largest alterations. T-cells are of pivotal importance for the generation of cell-mediated immunity. Cell-mediated immunity is a process that requires (1) recognition of antigens, (2) cell activation and proliferation, and (3) effector functions such as cellular cytotoxicity, phagocytosis, and immunoglobulin synthesis. Steps 2 and 3 seem to be particularly impaired during aging. Following antigen recognition, lymphocytes need to divide into several clones in order to mount effective cell-mediated immune responses. Cell division or proliferation can be readily assessed in vitroby stimulating lymphocytes with mitogens. When diseased subjects are excluded, immunosenescence involves impaired humoral

responses and blunted T-cell proliferation to mitogens (Pawelec et al. 2002). The latter is one of the most documented age-related change observed during aging (Liu et al. 1997; Murasko et al. 1987). Yet, these changes are not exclusive of aging and stress or GC treatment are also associated with decrements of T-cell proliferation) (see Table 2). Indeed, we have observed that healthy SENIEUR elders were significantly more distressed, had activated HPA axis and had significant lower (-53.6%) T-cell proliferation compared to young adults (Luz et al. 2006) (Fig. 2). Interestingly, the HPA axis may be implicated with this change since salivary cortisol levels were found negatively correlated to T-cell proliferation.

Thymic involution is a common consequence of mammal aging and it precedes the malfunctioning of the immune system, resulting in a diminished capacity to generate new T-cells. This thymic involution has been proposed to be due to changes in the thymic microenvironment resulting in its failure to support thymopoiesis (Henson et al. 2004). However, stress-related GCs (Selye 1936) or GC treatment (Fauci 1975) also atrophy the thymus and, to a lesser extent, other lymphoid tissues, triggering apoptotic death in immature T- and B-cell precursors and mature T-cells (Sapolsky et al. 2000). Therefore, thymic involution is not an exclusive phenomenon of aging.

The effector phases of both innate and acquired immunity are in large part mediated by cytokines. Different subpopulations of CD4+ T-cells synthesize specific cytokines and have been designated Th1 (IFN-g, IL-2, lymphotoxin a) or Th2 (IL-4, IL-10) cells. Th1 cytokines provide help for cell-mediated responses and the IgG2a antibody class switching whereas Th2 cytokines help B cells and IgA, IgE and IgG1 antibody class switching. Both human and mouse models have demonstrated that aging is associated with a Th1 to Th2 shift in cytokine production (Ginaldi et al. 2001; Globerson and Effros 2000). However, this is not an age-specific phenomenon but also seen during stress (Biondi 2001; Glaser et al. 2001) or GC treatment (Galon et al. 2002; Ramirez et al. 1996).

Recent work suggests that cytokines and hormones could be considered as possible links between endocrinosenescence and immunosenescence (Straub et al. 2000). Indeed, it has long been known that proinflammatory cytokines can readily activate the HPA axis during infection in animals (Besedovsky et al. 1977) or after administration in humans (Mastorakos et al. 1993). Another studies have linked the

Table 2 Changes in cell-mediated immunity. Direction of arrows indicate increase (⇧), decrease (⇩) or no change (⇔) compared to corresponding control levels. Based on references (Biondi 2001; Galon et al. 2002; Globerson and Effros 2000; Ramirez et al. 1996; Sapolsky et al. 2000)

Mechanism	Aging	Stress	GC treatment
Thymus	⇩	⇩	⇩
T-cell proliferation	⇩	⇩	⇩
Cytotoxicity	⇩	⇩	⇩
IL-2, IFN-γ	⇩	⇩	⇩
IL-4, IL-10	⇧	⇧	⇧
TNF-α, IL-1, IL-6	⇧ or ⇔	⇧	⇩

age-related decline in DHEA production to increased serum levels of IL-6 (Daynes et al. 1993; Straub et al. 1998). In addition, increased plasma TNF-α levels were correlated to major depression in the elderly (Vetta et al. 2001). However, we do not know exactly how the extent of these changes may be related to altered psychological and HPA axis functions in the elderly.

We have investigated whether psychoneuroendocrine status of healthy elders was associated with changes in lipopolysaccharide (LPS)-induced monocyte production of proinflammatory cytokines (TNF-α and IL-6) and soluble IL-2 receptor (sIL-2Ra) production by T-cells in vitro(Luz et al. 2003). Cellsofhealthyelders produced equivalent proinflammatory cytokines and soluble IL-2Rα when compared to cells of young adults. These data are in disagreement with previous work showing that human aging was associated to increased serum (Straub et al. 1998) or monocyte proinflammatory cytokines (Fagiolo et al. 1993; Gabriel et al. 2002). However, these data should be interpreted with caution because other cellular sources than monocytes can produce cytokines and thus increase serum levels. Considering that our cohort of elderly subjects was significantly distressed, we hypothesize this could have normalized the cytokines investigated in this study—due to antiinflammatory GC actions. On the other hand, there is also some evidence of increased proinflammatory cytokines during major depression (Schiepers et al. 2005; Trzonkowski et al. 2004; Vetta et al. 2001). Therefore, it becomes difficult to dissociate the cytokine changes observed in the elderly with those induced by psychological stimuli. Ghrelin, an endogenous ligand of the GH secretagogue receptor, has been recently demonstrated to inhibit the expression and production of proinflammatory cytokines (TNF-α, IL-1β and IL-6) (Dixit et al. 2004). This effect was mediated via binding on ghrelin receptors expressed on peripheral T-cells and monocytes. There is some evidence for increased stomach ghrelin production in the aged rat (Englander et al. 2004). Increased peripheral ghrelin levels may thus attenuate cytokine levels during aging. It remains to be investigated, however, whether psychological stress is capable of producing significant effects on stomach or immunoreactive ghrelin levels.

Previous studies have long demonstrated that serum growth hormone (hGH) levels are significantly reduced during aging (Corpas et al. 1993)—a process known as somatosenescence. However, hGH is not exclusively produced by pituitary gland and human immune cells are able to secrete several neuropeptides including GH (Hattori et al. 1994; Weigent et al. 1988). Immunoreactive GH has several immuno-enhancing proprieties and may be important in modulating both humoral and cellular immune function (Malarkey et al. 2002; Weigent et al. 1988). However, there is no data on the impact of aging on the production of GH by immune cells. In a recent study, we investigated whether somatosenescence could be associated with (a) related reduced production of immunoreactive GH and (b) psychological status of healthy SENIEUR elderly subjects (Luz et al. 2006). We found that elders had significantly lower (77%) serum hGH levels compared to young adults. In contrast, however, no changes in hGH production by activated monocytes or lymphocytes were observed between elders and adults (Luz et al. 2006). Interestingly, psychological distress (stress, anxiety and depression) was found negatively correlated to serum hGH levels only. No differences in serum hGH levels were

observed between groups when controlling for psychological variables (partial correlation). We provided first line of evidence that age-related psychological distress may be implicated with somatosenescence. Finally, somatosenescence was not associated with reciprocal decline in immunoreactive GH.

5 Role of DHEA During Immunosenescence

The lack of appropriate DHEAS levels during aging could be another detrimental factor for immunosenescence. This androgen and its metabolites have reported immune enhancing properties in contrast to the immunosuppressive action of GCs. Indeed, this hormone may be considered as natural antagonist of GCs and the impaired DHEA secretion, together with the increase of cortisol, results in an enhanced exposure of lymphoid cells to the deleterious GC actions. Therefore, previous studies have evaluated the immunomodulatory DHEA(S) effects in vitroas well as its properties during in vivosupplementation. The immunomodulatory in vitroeffects include increased mitogen-stimulated IL-2 production (Daynes et al. 1990; Suzuki et al. 1991), increased rodent or human lymphocyte proliferation (Padgett and Loria 1994), stimulated monocyte-mediated cytotoxicity (McLachlan et al. 1996), diminished TNFa or IL-6 production (Di Santo et al. 1996; Straub et al. 1998) and enhanced natural killer cell activity (Solerte et al. 1999).

DHEA(S) replacement therapy has yielded significant beneficial effects for healthy elders, including increased well-being, memory performance, bone mineral density and altered immune function (Buvat 2003). It has been shown that DHEA supplementation significantly increased NK-cell counts and activity and decreased IL-6 production and T-cell proliferation of the elderly (Casson et al. 1993). These data highlight the potential use of DHEA(S) as antiaging hormone. However, there is lacking information concerning the clinical significance of those findings.

Because of its enhanced immunomodulatory properties, several studies investigated the potential of DHEA(S) as adjuvants in vaccine preparations. Initial studies reported increased adjuvant effects on the immunization of aged mice with recombinant Hepatitis B surface antigen (Araneo et al. 1993) or influenza (Danenberg et al. 1995). These studies reported increased antibody titers to vaccines or even effective protection against challenge with the influenza infection (Danenberg et al. 1995). More recently, we studied the adjuvant effects of DHEAS during immunization to *Mycobaterium tuberculosis* in mice (Ribeiro et al. 2007). Only young mice co-immunized with *M. tuberculosis* heat shock protein 70 (HSP70) and DHEAS showed an early increase in specific IgG levels compared to old mice. However, splenocytes of both young and old mice that received DHEAS showed increased IFN-g production following priming in vitro with HSP70. These data further highlight the importance of DHEAS as hormonal adjuvant because of the role of this cytokine in the cellular response against mycobacteria. However, these animal data are in contrast to previous studies reporting DHEA(S) with minor (Degelau et al. 1997) or no adjuvant effects (Ben-Yehuda et al. 1998; Danenberg et al. 1997; Evans et al. 1996) dur-

ing immunization to influenza or tetanus in human elderly populations. Therefore, extrapolation from studies on murine models to the human should be regarded with caution—especially because of lower circulating DHEA(S) levels in rodents.

6 Aging Impairs Neuroendocrine-Immunoregulation

Most GC effects on the immune system are mediated via intracellular GC receptors (GR; genomic action) (McEwen et al. 1997). However, high concentration of GCs may also interact with membrane binding sites at the surface of the cells (nongenomic action) (Gold et al. 2001). The presence of these receptors indicates that the immune system is prepared for HPA axis activation and the subsequent elevation in endogenous GCs. However, the functional effect of a stress hormone will depend on the sensitivity of the target tissue for that particular hormone. For instance, the number and activity of specific receptors for these signaling molecules on the target organ will ultimately direct the physiologic effect of the stressor.

Recent findings suggest that GC sensitivity (a) may vary between different target tissues in the same organism, (b) shows large individual differences and (c) can be acutely changed in times of acute stress (Hearing et al. 1999; Rohleder et al. 2003). Furthermore and of special interest of this review, (d) GC sensitivity also changes during human ontogeny. Kavelaars and col. (1996) have shown that cord blood T-cells of newborns appear to be extremely sensitive to inhibition of the proliferative response. This high sensitivity of cells to DEX) can still be observed in the first two weeks after birth. Subsequently, the sensitivity to DEX inhibition of T-cell proliferation gradually decreases. At one year of age, the adult response pattern has been acquired. It is interesting that the increased sensitivity of the immune system to GC inhibition occurs at a period in life when the endogenous levels of glucocorticoids are low (Sippell et al. 1978). The increased sensitivity to glucocorticoids may serve as a compensatory mechanism, so that the important regulatory function of glucocorticoids is fully maintained despite low circulating levels.

In a recent study, we have also investigated the lymphocyte sensitivity to both synthetic (DEX) and natural occurring steroids (cortisol and DHEA) and so examined whether aging was associated with alterations in neuroendocrine-immunoregulation (Luz et al. 2006). It was found that healthy (SENIEUR) elders had a reduced (-19%) in vitrolymphocyte sensitivity to DEX (but not cortisol or DHEA) when compared to young adults. This phenomenon has previously been described during chronic stress (Bauer et al. 2000; Rohleder et al. 2002), major depression (Bauer et al. 2002; Bauer et al. 2003; Truckenmiller et al. 2005) or in clinical situations where GCs are administered, including treatment of autoimmune diseases, organ transplantation, and allergies. It has been recently shown (Rohleder et al. 2002) that aging is associated with changes in GC sensitivity of proinflammatory cytokine (TNF-α and IL-6) production following psychosocial (TRIER) stress test (Kirschbaum et al. 1993). In particular, monocytes of healthy (non-SENIEUR) eld-

erly men had a higher sensitivity to DEX treatment in vitroat baseline and showed a reduced sensitivity to this steroid following acute stress exposure (speech coupled to mental arithmetic task). These data suggest that psychological factors may be implicated in regulating peripheral GC sensitivity during healthy aging.

A reduced sensitivity to GCs can also be demonstrated at the central level during aging. Indeed, higher cortisol levels in old than in young subjects have been described during some pharmacological challenges, such as the DEX suppression test, the stimulation by human or ovine corticotrophin-releasing hormone or by physostigmine (Ferrari et al. 2001; Raskind et al. 1994).

6.1 Potential Mechanisms of Impaired GC Signaling

The mechanisms underlying acquired steroid resistance are poorly understood. Based on our previous observations (Luz et al. 2003) we suggest that higher cortisol levels would render lymphocytes to be less sensitive to the effects of GCs in vitro. Indeed, there is some evidence in the literature suggesting that changes in GC sensitivity could be the result of chronic GC treatment (de Kloet et al. 1998; Silva et al. 1994). Several mechanisms may be implicated in this acquired steroid resistance (Juruena et al. 2003; Rohleder et al. 2003). Fig. 3 summarizes putative molecular mechanisms that may account for age-related changes in GC sensitivity. There is some evidence that aging is associated with reduced numbers of intracellular GRs (Grasso et al. 1997; Zovato et al. 1996) but changes in GR affinity cannot be ruled out. In addition, altered translocation of GC/GR complex to nucleus and altered acti vity of transcription factors may also explain acquired GC resistance. Alternatively, it has been shown that a non-ligand binding β-isoform of the human GR (hGRβ) may also be implicated in acquired steroid resistance (Castro et al. 1996). It was hypothesized that the hGRβ probably heterodimerises with ligand-bound hGRα and translocates into the nucleus to act as a dominant negative inhibitor of the classic receptor. However, there is no evidence for age-related changes in expression of GR isoforms. Furthermore, we cannot exclude the participation of mutations in the GR or changes in the GR transduction system (e.g., altered AP-1 and NF-kB expression, heat shock proteins) in promoting tissue sensitivity to glucocorticoids (reviewed in Bronnegard et al. 1996).

In addition, there is considerable evidence that cytokines may have a significant impact on GR expression and function. There is some evidence suggesting that local concentrations of cytokines produced during an inflammatory response may produce acquired GR resistance (Pariante et al. 1999a). Of note, the GR resistance in major depression has been associated with increased levels of proinflammatory cytokines (TNF-α, IL-1 and IL-6) and acute phase proteins (Maes et al. 1993; Schiepers et al. 2005; Trzonkowski et al. 2004). Furthermore, it has recently been shown that IL-13, a cytokine with similar properties to IL-4, reduces GR binding affinity in peripheral blood mononuclear cells (PBMCs) (Spahn et al. 1996). In summary, various mechanisms may mediate age-related changes in immune GC signaling, however, further research is required to fully understand the basis of the changes in altered lymphocyte sensitivity to steroid.

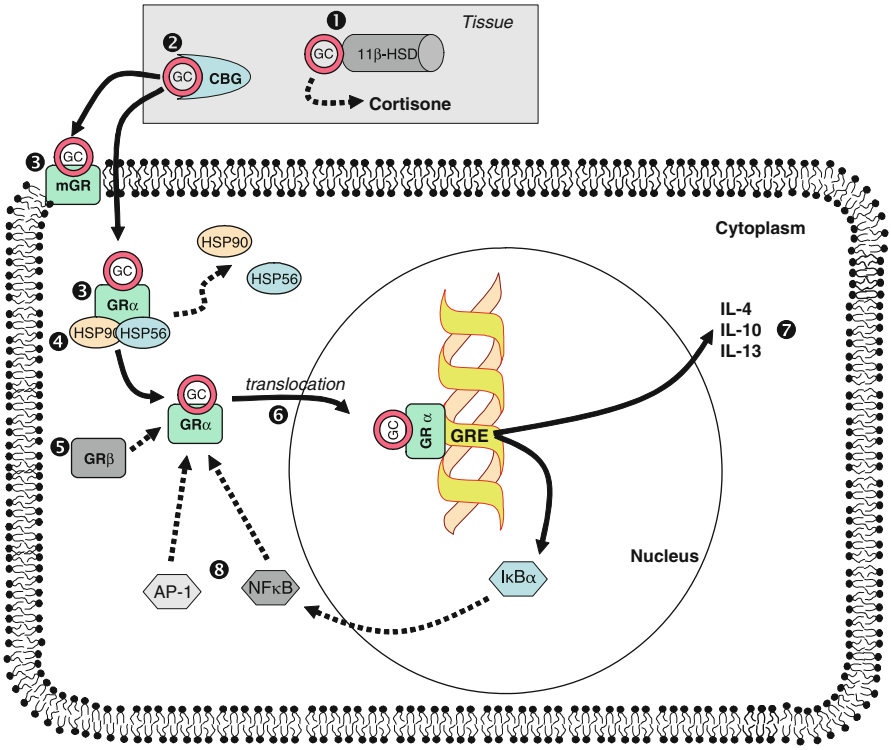

Fig. 3 Cellular sensitivity to glucocorticoids. Extracellular hormone availability can be determined by (1) differential expression of tissue-dependent expression of 11β-hydroxysteroid dehydrogenases that catalyze the interconversion of active glucocorticoids (cortisol) to inactive forms (cortisone) and vice versa (Zhang et al. 2005); and (2) levels of plasma corticosterone binding globulin (CBG) which delivers biologically active glucocorticoids (GCs) into peripheral tissues. Intracellular sensitivity to glucocorticoids can be modulated by several mechanisms, including: (3) altered densities of functional membrane or intracellular glucocorticoid receptor (GRα) as well as receptor affinity changes (Pereira et al. 2003); (4) altered expression of heat shock proteins (HSP90 and HSP56) which stabilizes GR and are dissociated following binding of GCs (Picard et al. 1990); (5) altered expression of GRβ which in turn antagonises GRα (Castro et al. 1996); (6) altered translocation of GR-GC complexes into the nucleus (Matthews et al. 2004); (7) altered expression of several cytokines (Kam et al. 1993; Pariante et al. 1999b); and (8) altered expression of transcription factors AP-1 (Adcock et al. 1995) and NFkB which in turn antagonise GRα. Dashed lines represent inhibitory actions on GRα Adapted from Bauer (2005).

7 The Impact of Chronic Stress on Strictly Healthy Aging—Damaging and Protecting Effects

The caregiving of demented patients is a recognized model to study the impact of chronic stress in elderly populations (Bauer et al. 2000; Kiecolt-Glaser et al. 1991; Vedhara et al. 1999). Care of the chronically ill is a demanding task that is associated with increased stress, depression, and poorer immune function (Redinbaugh

et al. 1995). Furthermore, providing care for a relative with dementia typically falls on the partners who are themselves elderly and often ill prepared for the physical and emotional demands placed upon them.

The daily stress experienced by the caregivers of Alzheimer patients may accelerate many age-related changes, particularly on neuroendocrine and immune systems. We have previously demonstrated that caregivers of demented patients had a blunted T-cell proliferation in association with increased cortisol levels (Bauer et al. 2000) compared to nonstressed elders. Furthermore, lymphocytes of elderly caregivers were more resistant to GC treatment in vitrocompared to noncaregiver elders. When stressed elderly are compared to healthy elderly and young adults (see Fig. 4), these immunological changes are found in similar magnitude to increased cortisol levels. These data suggest that chronic stress and cortisol would thus accelerate human immunosenescence. Indeed, it has recently been observed that psychological stress (both perceived stress and chronicity of stress) was significantly associated with higher oxidative stress, lower telomerase activity, and shorter telomere length, which are known determinants of cell senescence and longevity (Epel et al. 2004).

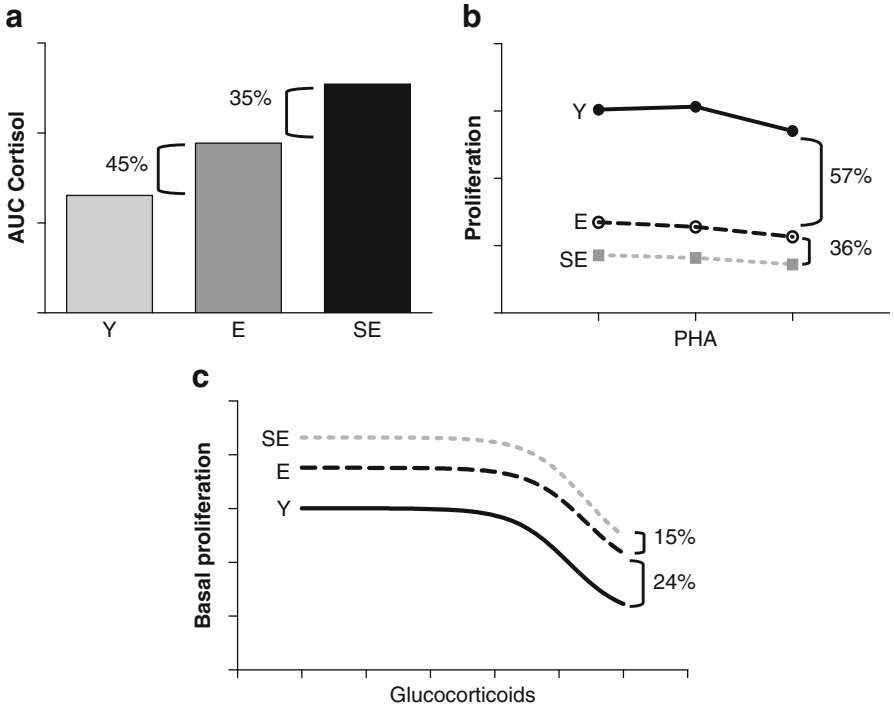

Fig. 4 Effects of chronic stress on cortisol and T-cell function during aging. Young adults (Y), elderly (E) or stressed elderly (SE) subjects were compared accordingly to area under the curve (AUC) cortisol production (A), T-cell proliferation to phytohemagglutinin(PHA) stimulation (B) or T-cell sensitivity to glucocorticoids in vitro(C). Data summarized from previous work (Bauer et al. 2000; Luz et al. 2003; Luz et al. 2002) and shown as the percentage of change between groups. Adapted from Bauer (2005).

Several studies have implicated caregiving as a risk factor for health of elderly populations. Compared with noncaregivers, subjects who provide care to a spouse with a stroke or dementia report more infectious illness episodes (Kiecolt-Glaser et al. 1991), they have poorer immune responses to influenza virus (Kiecolt-Glaser et al. 1996; Vedhara et al. 1999) and pneumococcal pneumonia vaccines (Glaser et al. 2000), they present a slow wound healing (Kiecolt-Glaser et al. 1995), they are at greater risk for developing mild hypertension (Shaw et al. 1999), and they may be at greater risk for coronary heart disease (Vitaliano et al. 2002). In addition, a prospective longitudinal study found that the relative risk for mortality among caregivers was significantly higher (63%) than noncaregiving controls (Schulz and Beach 1999). A recent study indicates that a proinflammatory cytokine (IL-6) may be involved with this increased morbidity in caregiving populations (Kiecolt-Glaser et al. 2003). It remains to be investigated, however, how the extent of these changes may be related to neuroendocrine alterations observed during aging.

Recent data produced by our laboratory have suggested that the maintenance of health status during aging may protect elders from chronic stress exposure. We have recruited strictly healthy (SENIEUR) elderly caregivers (n=41) from a large population of primary caregivers of demented patients (n=342). Only 12% of caregivers were considered "strictly healthy" accordingly to this stringent protocol and this may further confirm that chronic stress exposure is associated with increased morbidity in elderly populations. Therefore, we investigated whether a stringent health status would protect caregivers from chronic stress exposure and compared psychoneuroendocrine and immunological changes to nonstressed controls.

We observed that SENIEUR elderly caregivers were significantly distressed, as shown by increased stress, anxiety and depression scores as well as by higher systolic blood pressure compared to nonstressed elders (unpublished data). These data provide further support for this chronic stress model. However, salivary cortisol levels remained unchanged in healthy caregivers compared to nonstressed elders, contrasting to previous work (Bauer et al. 2000). Indeed, previous studies have linked the stress-related hypercortisolemia with blunted cellular and humoral immune responses (Bauer et al. 2000; Vedhara et al. 1999). This could be of beneficial value for the caregiver and may indicate that a stringent health status in the elderly can buffer the impact of chronic stress on neuroendocrine responses. Therefore, healthy caregivers would be protected from the deleterious effects of cortisol excess in the organism. The normalization of HPA axis function could be related to endocrine habituation associated to the development of coping strategies, cognitive and learning skills (Huether 1996). These results, taken together with our previous studies with nonstressed SENIEUR elders, may further indicate that a stringent health status may protect individuals from stress exposure but not from age-related increase in salivary cortisol (Luz et al. 2003). The peripheral lymphoid cells could be spared from the increased and deleterious cortisol signaling normally observed during chronic stress exposure.

The SENIEUR caregivers had increased T-cell proliferation when compared nonstressed healthy controls (unpublished data). We speculate that the intact HPA axis function may have spared the lymphocytes from the negative effects of cortisol excess. Peripheral lymphocytes of caregivers are thus expected to display a better GC signal-

ing. Indeed, the lymphocytes of SENIEUR caregivers had a higher GC sensitivity when compared to non-stressed controls, as shown by the increased GC-induced suppression of lymphocyte proliferation in vitro(unpublished data). These data further highlight the close communication of neuroendocrine and immune systems during aging. In contrast to increased peripheral GC-immune signaling, the healthy caregivers were more resistant to central effects of glucocorticoids. Indeed, there were a higher proportion of SENIEUR caregivers (29.3%) who had failure to suppress cortisol levels through dexamethasone administration comparing to nonstressed controls (3%). The dexamethasone suppression test suggests that caregivers may have a dysfunction of the HPA axis related to chronic stress exposure but not to peripheral GC levels. These data are in partial contrast to previous work relating hypercortisolemia to reduced lymphocyte sensitivity to GCs in elderly British caregivers (Bauer et al. 2000). However, the central defect in HPA axis regulation may not necessarily be associated to endogenous GC levels since previous studies reported this change in patients with major depression without hypercortisolemia (Bauer et al. 2003).

Taken together, these results suggest that a strictly healthy (SENIEUR) aging may buffer or attenuate many deleterious neuroendocrine and immunological effects associated to chronic stress exposure.

8 The Psychoneuroendocrine Hypothesis of Immunosenescence

The studies reviewed here support the notion that immunological changes observed during healthy aging may be closely related to both psychological distress and stress hormones. Of note, changes cellular trafficking as well as cell-mediated immunity observed during aging are similarly found following stress or chronic GC exposure. These changes are mainly produced via engagement of specific intracellular adrenal receptors expressed on peripheral lymphocytes. Based on these data, the neuroendocrine hypothesis of immunosenescence is reconsidered here (see Fig. 5). During aging, cumulative neuronal damage produced by stress-related cortisol action in the brain (hippocampus and hypothalamus) is associated with decreased central sensitivity to cortisol (Ferrari et al. 2001; Raskind et al. 1994; Sapolsky et al. 1986). This will lead to increased cortisol levels (Deuschle et al. 1997; Ferrari et al. 2004; Halbreich et al. 1984; Heuser et al. 1998; Luz et al. 2003; Van Cauter et al. 1996) which in turn may produce more neuronal damage in the brain and promote thymic involution. These effects may be exacerbated by reduced DHEA/DHEAS levels frequently observed during aging. The impaired DHEAS secretion, together with the increase of cortisol, results in an enhanced exposure of various bodily systems (including brain and immune system) to the cytotoxic/immunomodulatory effects of GCs. These tissues are preferentially targeted by cumulative cortisol action because they express the greatest densities of MRs (hippocampus) and GRs (thymus) (McEwen et al. 1997). The critical consequence of thymic involution is reduced output of naïve T-cells—a hallmark of immunosenescence. It remains to be investigated, however, why peripheral T-cells are preferentially targeted during aging comparing to B or NK-cells. It

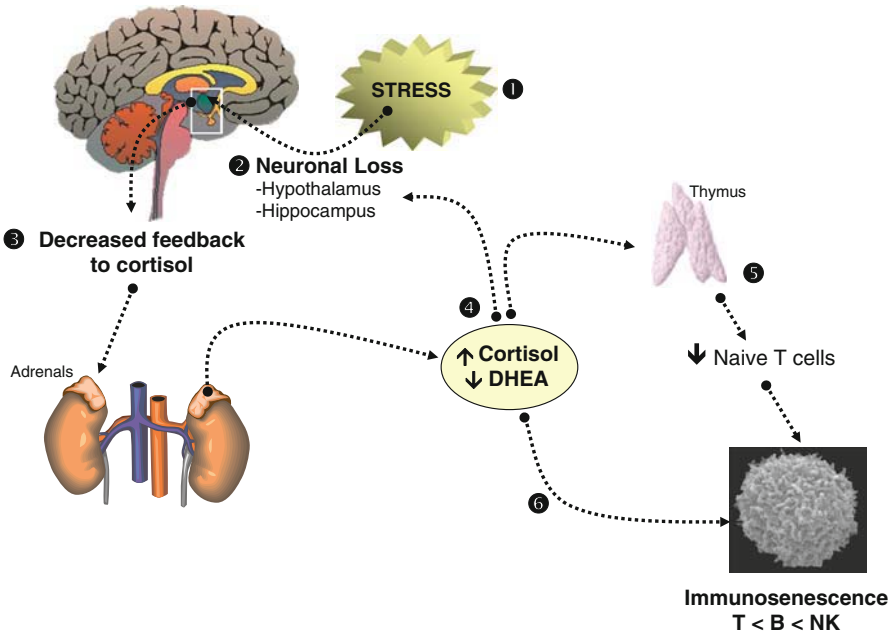

Fig. 5 The psychoneuroendocrine hypothesis of immunosenescence. During aging, cumulative neuronal damage produced by stress-related cortisol action in the brain (1 and 2) (Sapolsky et al. 1986) is associated with decreased central sensitivity to cortisol (3) (Ferrari et al. 2001; Raskind et al. 1994). This specific effect is associated with increased cortisol/DHEA ratio (4) (Ferrari et al. 2004; Luz et al. 2003) which in turn may produce more neuronal damage in the brain and further promote thymic involution (5). The latter may be related to immunosenescence via two ways: (a) indirectly reducing the output of central naïve T-cells and (b) directly acting at the level of peripheral lymphoid cells (6)(Luz et al. 2006). Adapted from Bauer (2005).

should be kept in mind this hypothesis is over simplistic and do not take into account other stress-related mediators (neuropeptides, noradrealine, GH, etc.) and intrinsic cellular mechanisms of aging, including oxidative stress and telomere shortening. Further studies are required to investigate whether cellular aging is associated with aging of neuroendocrine functions. In addition, the role of increased cortisol/DHEA ratio during immunosenescence may be over simplistic since many other important hormones also become lower during aging in relation to cortisol (Straub et al. 2001).

9 Conclusions and Outlook

When age-related diseases are controlled for, healthy aging is associated with changes in allostatic systems (endocrine and immune) that play major roles in the adaptation of organism to outside forces that are threatening the homeostasis of the internal milieu. In particular, healthy aging is associated with significant psychological distress and activation of the HPA axis (increased cortisol and reduced DHEA).

Over weeks, months, or years, exposure to increased secretion of stress hormones would result in allostatic load ("wear and tear") and its pathophysiologic consequences (McEwen 1998). Given the findings that even discrete HPA axis activation may impair cognitive function (Lupien et al. 1994) and induce sleep disturbances (Starkman et al. 1981), conditions frequently associated in the elderly, psychological or pharmacological strategies attenuating or preventing increased HPA function during aging might be of considerable benefit for the elderly.

Although the mechanisms underlying immunosenescence are still being unraveled, it is becoming increasingly clear that many of the physiologic changes associated with aging are characterized by deficient communication between neuroendocrine and immune systems. Data presented here suggest that aging is associated with reduced lymphocyte sensitivity to GCs. Glucocorticoid-induced acquired resistance may have an important physiological significance of protecting cells from the dangerous effects of prolonged GC-related immunosuppression. However, the significance of this adaptive phenomenon is questionable since T-cell proliferation is still profoundly suppressed during aging. Additionally, altered steroid immunoregulation may have important therapeutic implications in clinical situations where GCs are administered, including treatment of autoimmune diseases, organ transplantation, and allergies. Clinicians should consider both patient's age and psychological status in prescribing steroids as anti-inflammatory drugs.

Chronic stressed elderly subjects may be particularly at risk of stress-related pathology because of further alterations in GC-immune signaling. Elderly individuals who experience chronic stress exhibit poorer immune functions, and thus increased disease vulnerability, than their less stressed counterparts. Indeed, chronic stressed elderly populations are associated with increased morbidity and mortality rates. Therefore, stress management and psychosocial support should promote a better quality of life for the elderly as well as reducing hospitalization costs for the governments. In addition, the maintenance of health status during aging may protect elders from chronic stress exposure (Fig. 6). Further studies in systems biology are needed to analyze the role and relationships of health-related behaviors on immunity that might promote better coping with aging and stress exposure. We are currently entering a new era of investigation in biology of aging in which systemic approach will replace reductionism in order to explain how we age and get sick.

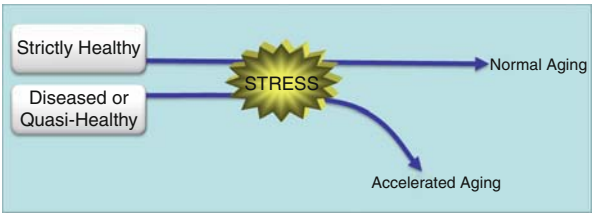

Fig. 6 Buffering effects of health status during chronic stress exposure. This picture presents two different scenarios of protective (upper line) or damaging (bottom line) stress-related effects during aging. Strictly healthy individuals will be protected from chronic stress and will have extended life span. Diseased or quasi-healthy subjects, however, will show accelerated aging.

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Thymus

Thymic Involution and Thymic Renewal

Frances T. Hakim

Contents

Abbreviations

AIRE	Autoimmune regulator
CCL	Chemokine (C-C motif) ligand; CCL21 and CCL25
CCR	Chemokine (C-C motif) receptor; CCR9 and CCR7
CMJ	Cortical-medullary junction
cTEC	Cortical thymic epithelial cells
DN	Double negative ($CD4^-$ $CD8^-$) thymocyte
DP	Double positive ($CD4^+CD8^+$) thymocyte
ETP	Early thymocyte progenitor
FGF	Fibroblast growth factor
FGFR2-IIIb	Fibroblast growth factor receptor 2-IIIb
GH	Growth hormone
HAART	Highly active anti-retroviral therapy
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor 1
IL-7	Interleukin 7
KGF	Keratinocyte growth factor, also FGF7
LHRH	Luteinizing hormone releasing hormone
MPP	Multipotent progenitor cell
mTEC	Medullary thymic epithelial cells
PSGL1	P-selectin ligand
PVS	Perivascular space

F. T. Hakim (✉)
Experimental Transplantation and Immunology Branch
National Cancer Institute, NIH
10 Center Drive, CRC-3E-3330
Bethesda, MD, 20892-1203
Tel: 1 301-402-3627
Fax: 301-480-8146
E-mail: hakimf@mail.nih.gov

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RAG2/γ_c	Recombination-activating gene/common γ chain
RTE	Recent thymic emigrant
SCZ	Subcapsular zone
sjTREC	Signal joint T cell receptor rearrangement excision circle
SP	Single positive ($CD4^+$ or $CD8^+$) thymocyte
TCR	T cell receptor
Treg	Regulatory T cell
VDJ	Variable, diversity and joiner elements of the TCR beta chain

Abstract: A primary factor in immunosenescence, the age-dependent deterioration in immune function, is the decline in the capacity to generate naïve T-cells due to thymic involution. The thymus reaches its greatest size and cellularity in the first year of life and undergoes a gradual involutional decline in both structure and thymopoietic productivity. Thymic involution results from the interplay of systemic factors and intrinsic changes in thymic epithelial cells and thymocyte progenitors themselves. In patients undergoing lymphodepletion, however, the thymus is capable of significant renewal through the fifth decade of life. This chapter will explore the factors regulating thymic growth, involution and renewal.

Keywords: Thymus • Thymocyte • Thymic epithelial cells • Cortex • Medulla • Hematopoietic stem cell • ETP • DN • Notch • IGF-1 • KGF • IL-7

1 Introduction: The Immunologic Consequences of Thymic Involution in the Elderly

Aging is associated clinically with a decline in adaptive immune system responses to vaccines, increases in the frequency and severity of infectious diseases, and an increased incidence of chronic inflammatory and autoimmune disorders. The alterations in immune competence underlying these disorders have been collectively termed immunosenescence. A primary cause of immunosenescence is the gradual decline in thymic generation of new naïve T-cells. When the thymus can no longer replace the naïve T-cells lost daily, the result is a steady decline in the levels of naïve T-cells.

This decline has profound consequences for immune function. The naïve T-cell population provides a reservoir of T-cell receptor (TCR) diversity that may be needed to respond to novel antigens. In young and even middle aged adults the repertoire diversity has been estimated at 20 million different TCRβ chains; in the elderly (greater than 70 years), the pool has severely contracted to 200,000 TCRβ specificities (Naylor et al. 2005). The 99% decline in TCR repertoire diversity in CD4 T-cells in the elderly may in itself be a critical factor in limiting functional response; a 2–10 fold decrease in repertoire has been found to abrogate T-cell mediated responses in mice (Nanda et al. 1991). Furthermore, studies in adults recovering from lymphopenia have demonstrated that both the capacity to respond to vaccines and the resistance to opportunistic infections correlate with the levels of naïve $CD4^+$ T-cells and the presence of broad TCR repertoire diversity (Lewin et al. 2002; Roux et al. 2000).

Since naïve cells provide the reservoir from which memory cells are drawn, a decline in the frequency of naïve cells impacts memory populations. The relative representation of different T-cell specificities remains relatively constant throughout life, due to a homeostatic balance between turnover and a steady influx of new T-cells (Tanchot et al. 2000). In the elderly, this balance is lost. The repertoire diversity of memory populations in the elderly declines concurrent with the decline in the frequency of naïve cells (Schwab et al. 1997). Analogously, the repertoire diversity of the memory $CD4^+$ T-cell population in adult patients recovering from transplant-induced lymphopenia has been found to directly depend upon the extent of posttransplant thymic function (Hakim et al. 2005). Individuals lacking a strong recovery of thymic function had a limited oligoclonal repertoire in their memory T-cell populations even 2–5 years post transplant (Hakim et al. 2005). Chronic infection with CMV and to a lesser extent EBV may further alter the memory/effector repertoire by driving virus-reactive cells into oligoclonal expansions or even to replicative exhaustion (Fletcher et al. 2005). Oligoclonal expansions can by themselves limit overall immune function in the remainder of the T-cell population (Khan et al. 2004; LeMaoult et al. 2000; Messaoudi et al. 2004). Yet these expanded cells are often dysfunctional, responding poorly to stimulation by their target antigens (Ouyang et al. 2003). Thus the loss of a strong influx of 'replacement' cells into the memory/effector pool may contribute to immune deficits.

The decline in naïve $CD4^+$ T-cell numbers may also affect humoral immune function. Much of the decline in vaccine responses in the elderly is due to reductions in the formation and function of germinal centers as compared to those in young individuals (Lazuardi et al. 2005). Germinal center formation depends upon the frequency of CCR7 and CD62L-bearing naïve and central memory $CD4^+$ T-cells, which can enter lymph nodes and initiate germinal center formation. These CD4 populations decline in the elderly. Within germinal centers, cognate interaction between $CD4^+$ T-cells and B-cells promotes somatic hypermutation of immunoglobulin chains, a process that increases the avidity of antibodies. Age-dependent deficits in $CD4^+$ T-cells that reduce cognate B-T interactions may therefore contribute to the decline in antibody avidity observed in the elderly.

Finally the involutional changes in the thymus with age may result not only in immune deficits but also in dysfunctional increases in autoreactivity. The thymus contributes to the regulation of tolerance and the prevention of autoimmunity at many levels. First of all, auto-reactive $CD4^+$ and $CD8^+$ T-cells are clonally deleted during negative selection in the thymus, establishing central tolerance. The unique expression of the AIRE (autoimmune regulator) gene in medullary thymic epithelial cells (mTEC) results in expression of a broad array of tissue-specific antigens (Gallegos et al. 2004). Thymocytes bearing T-cell receptors (TCR) that bind to these tissue-specific antigens are clonally deleted. This process removes self-reactive T-cells from the repertoire before T-cells are exported into the periphery. Although the thymus is known to continue to support a low level of thymopoieis for many decades (Jamieson et al. 1999; Sempowski et al. 2000), the continued efficiency of negative selection in involuted thymuses has not been evaluated in man. With age, there is a decline in the level of mTEC expressing high levels of MHC II (Gray

et al. 2006); these include the AIRE expressing cells critical in negative selection. Secondly, regulatory T-cells (Treg) are believed to play a critical role in the prevention of autoimmunity, suppression of inflammatory responses and the modulation of T-cell homeostasis. Treg develop in parallel with $CD4^+$ and $CD8^+$ effector T-cells in the thymus (Wing et al. 2002; Wing et al. 2005), but whether their production similarly declines in parallel with overall thymopoiesis has not been assessed. Since Treg development has been linked to Hassall's corpuscles of the human thymus (Watanabe et al. 2005), the loss of these medullary structures with aging may be problematic. Treg can also arise in the periphery from memory CD4 T-cell populations in adults (Walker et al. 2003), but such cells may turn over rapidly (Vukmanovic-Stejic et al. 2006). The numbers of circulating Treg cells have been found to actually increase with age, but regulatory function declined (Gregg et al. 2005; Zhao et al. 2007). Finally, productive thymopoiesis, in and of itself, may be a factor deterring autoimmunity. Under conditions of lymphopenia prolonged by inadequate thympopoiesis, compensatory peripheral expansion of T-cells occurs to maintain stable T-cell levels. This extended homeostatic proliferation has been proposed to provide the opportunity for T-cells reactive to self-antigens to expand, leading to autoimmune disorders (King et al. 2004). Both lymphopenia and elevated levels of cycling (Ki-67^+) peripheral T-cells are found in the elderly, consistent with such a model of autoimmune development (Naylor et al. 2005). In all of these respects, the thymus maintains immunologic tolerance to self. The gradual age-dependent decline in thymic cytoarchitecture and thympoietic productivity may therefore contribute to the development of autoreactivity and loss of self-tolerance.

2 Thymic Organogenesis and Thymopoiesis

The thymus is located in the superior mediastinum, just over the heart, and consists of two lobes, connected by areolar tissue and enclosed in a fibrous capsule. Each lobe is further subdivided into lobules containing immature thymocytes in a network of epithelial cells termed thymic stroma. The denser outer areas are termed cortex and the looser inner areas are termed medulla. Committed T-progenitors enter the thymus through the vasculature at the cortico-medullary junction (CMJ); as these cells proliferate and differentiate, they follow a migration path outwards through the cortex to the subcapsular zone (SCZ) (Fig. 1). Thymocytes then migrate back inwards through the cortex to complete maturation and selection in the medulla, before emigrating from the thymus as mature, naïve T-cells via the CMJ vasculature. Lying between the lobules are is the perivascular space of the thymus (PVS). While limited to narrow septa of connective tissue in the neonate, it is the PVS which expands and fills with adipocytes and fibroblasts during thymic involution.

The thymus begins as an outpocketing of the third pharyngeal pouch endoderm which gives rise to thymic epithelial cells. Mesenchymal cells derived from neural crest contribute to the thymic capsule and PVS connective tissue at this early stage, but previously postulated ectodermal contributions to thymic anlage have recently

been excluded (Gordon et al. 2004). When the thymic rudiment is subsequently colonized by a wave of hematopoietic progenitors, the progenitors of the thymic epithelial cells are still immature and capable of differentiation into both cortical and medullary thymic epithelial cells (cTEC and mTEC) (Rossi SW et al. 2006; Rossi SW et al. 2007). Thymocytes are not necessary for the initial development of the TEC, but are required for the subsequent development and maintenance of TEC (Klug et al. 2002). Mesenchymal production of the fibroblast growth factor family cytokine FGF10 is necessary, however, for proliferation and early expansion of the TEC (Jenkinson et al. 2003; Jenkinson et al. 2007). Failure to express the FGFR2-IIIb receptor for these mesenchymally derived factors blocks TEC expansion (Revest et al. 2001). Thus it is through the cooperative interactions of mesenchymal cells, endoderm-derived thymic epithelial cells and hematopoietic-derived T-cell progenitors that the fetal thymus is formed.

T-progenitors arise in the marrow from hematopoietic stem cells (HSC). HSC give rise to multipotent progenitors (MPP), which in turn are the source of myeloid and lymphoid cells. Progenitors may become committed to the T lineage upon interaction of the Notch ligand Delta-like-1 on supportive stroma with Notch expressed on lymphoid progenitors (Schmitt et al. 2002; Schmitt et al. 2004). This commitment step may occur in the marrow prior to emigration to the thymus, but when the early thymocyte progenitor (ETP) engrafts in the thymus the T lineage commitment is reinforced by Notch/Notch ligand interactions during thymopoiesis (Schmitt et al. 2004). Upon leaving the marrow, T-progenitors home to the thymus (Rossi FM et al. 2005). The most immature thymocytes are termed double negative cells (DN) due to a lack of expression of CD4 or CD8. In the DN stage, subdivided into DN1 through DN4, thymocytes increase in number, migrate outward through the cortex, and rearrange the variable (V), diversity (D) and joiner (J) segments of the TCRβ chain (see Fig. 1). During the DN3 stage, signaling through the rearranged TCRβ chain and an associated invariant preTα receptor chain triggers the main proliferative expansion of thymocytes. and differentiation into CD4 and CD8 double positive (DP) cortical thymocytes. Upon final rearrangement of the TCRα chain and surface expression of a complete TCRαβ, the DP-cells undergo positive selection, based on affinity for Class II or Class I MHC molecules, into single positive (SP) $CD4^+$ or $CD8^+$ T-cells, respectively. During this positive selection process, DP thymocytes migrate back inwards across the cortex. Finally, within the medulla, the SP-cells undergo a negative selection process in which autoreactive cells are clonally deleted. Mature SP $CD4^+$ and $CD8^+$ T-cells then leave the thymus through the cortical-medullary vasculature (See Fig. 1).

Interactions between developing thymocytes and surrounding thymic epithelia control all aspects of thymopoiesis. Entry of T-progenitors depends on interaction between chemokine and adhesion molecules on progenitors, such as CCR9 and PSGL1, and the corresponding ligands (CCL25 and P-selectin) expressed by thymic stroma (Jenkinson et al. 2007; Rossi FM et al. 2005; Schwarz et al. 2007; Scimone et al. 2006; Zediak et al. 2007). T-cell lineage commitment and differentiation is reinforced upon signaling through Notch receptors (on T-progenitor cells) triggered by delta-1-like ligand expressed on stromal cells (Ciofani et al. 2004; Schmitt and

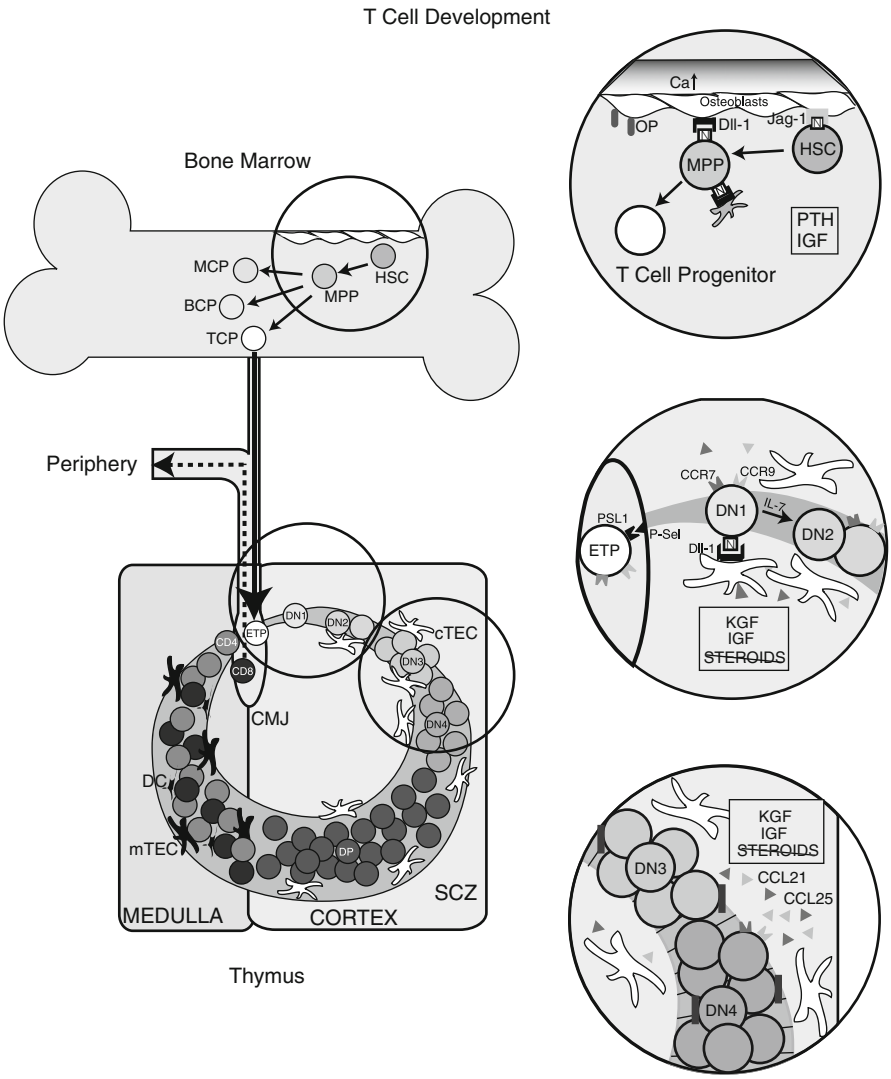

Fig. 1 An overview of T-cell maturation is shown at the left with expanded views on the right of the 3 areas of control of thymic productivity: the marrow compartment site of T-progenitor commitment, the CMJ vasculature and inner thymic cortex niches for progenitor engraftment, and the outer cortex and SCZ sites of thymocyte migration and expansion. Agents affecting each of these sites are noted in boxes.

In the bone marrow, pluripotent hematopoietic stem cells (HSC) with the capacity for self-renewal are supported in a calcium rich endosteal microenvironment. Proliferation is regulated by osteopontin (OP), secreted by osteoblasts, and by interaction of Notch (N, white box) on HSC with its ligand Jagged-1 on osteoblasts; osteoblast support for hematopoiesis in turn is regulated by levels of parathyroid hormone (PTH) and Insulin-like Growth Factor 1 (IGF) and IL-7. HSC give rise to Multipotent Progenitors (MPP), which cannot self-renew, but can expand and give rise to myeloid common progenitors (MCP), B-lymphoid and T-lymphoid progenitors. Commitment to the T

Zuniga-Pflucker 2002; Schmitt et al. 2004). Thymocyte migration across the thymus is controlled by chemokine signals in the stroma (Takahama 2006). Cytokines secreted by TEC, such as IL-7, support thymocyte survival at key checkpoints, such as the DN1–>DN2 transition (Phillips et al. 2004) and the positive selection process (Yu et al. 2003). Finally, thymic stroma is critical to the positive and negative selection processes. Cortical TEC expressing Class I and Class II MHC molecules support positive selection of cortical thymocytes into CD4 and CD8 SP T-cells. Medullary TEC and dendritic cells, expressing tissue antigens, support negative selection of autoreactive thymocytesand development of Treg (Gallegos and Bevan 2004; Watanabe et al. 2004). Thus all aspects of adult thymopoiesis depend upon interaction of thymocytes and TEC.

Yet this interaction is not unidirectional. After the fetal organogenesis period, the maintenance of TEC structure is strictly dependent upon on the presence of thymocytes. In mice in which the T-cell developmental pathway is blocked at its earliest stages, such as Ikaros mutant, CD3εtg26 mice and recombination-activating gene/common γ chain (RAG2/γ_c) deficient mice, immature TEC cells proliferate in the fetal thymus and develop characteristic cytokeratins (Jenkinson et al. 2005; Klug et al. 2002). The continued maintenance of cortical and medullary TEC in the adult, however, requires the presence of functional thymocytes. Thymuses in adult CD3εtg26 mice, in which T-cell development is blocked at the earliest DN1 stage, show an absence of both cortical and medullary TEC, with only immature TEC pro-

lineage results from Notch interactions with Delta-like ligand 1 (Dl1). T-progenitors leave the bone marrow and home to the thymus, where they become early thymocyte progenitors (ETP).

The thymus is divided structurally and functionally into three main areas, the subcapsular epithelial zone (SCZ), the cortex (COR) and the medulla (MED). Distinct populations of cortical and medullary thymic epithelial cells (cTEC and mTEC) support T-cell maturational stages in each region. The migration path of thymocytes undergoing proliferative expansion and differentiation is symbolized by the gray band.

T-progenitor cells enter the thymus from the circulation via the vasculature at the cortico-medullary junction (CMJ). Uptake of ETP into the thymus is in part controlled by the interaction of P-Selectin Ligand 1 (PSGL1, black) expressed on the ETP with P-selectin (P-sel, black triangle) on CMJ vasculature. Upon entering the cortex, the earliest thymocytes, termed double negative cells (DN) due to a lack of expression of CD4 or CD8, strengthen commitment to the T lineage by repeated signaling through Notch (N, white box) and Delta-like ligand 1 on cTEC, and migrate outward toward the subcapsular zone. Migration depends upon adhesion of DN integrins (black box) to VCAM (black stripes) and is polarized by a gradient of cTEC secreted chemokines, including CCL21 (gray triangle) and CCL25 (dark gray triangle) which bind to chemokine receptors CCR7 and CCR9 on DN-cells. Based on the expression of surface markers (that correlate with the process of TCRβ chain rearrangement), the DN stage is subdivided into DN1 through DN4. During the outward migration and in the SCZ, the main proliferative expansion of DN cells occurs. Several factors, such as KGF and IGF-1 and gonadal steroids have regulatory effects on thymopoiesis, increasing the uptake of T-progenitors, the numbers of TEC and the associated expansion of DN thymocytes. Upon final rearrangement of the TCRα chain and expression of a complete TCRαβ, the CD4 and CD8 double positive (DP) thymocytes begin a migration back across the cortex and into the medulla. DP differentiate into single positive (SP) $CD4^+$ or $CD8^+$ T-cells, based on affinity for Class II or Class I MHC molecules, respectively. Finally, within the medulla, the SP cells undergo a negative selection process in which autoreactive cells are clonally deleted based on interaction with self peptides expressed on mTEC and dendritic cells (DC). Mature SP $CD4^+$ and $CD8^+$ T-cells then leave the thymus through the CMJ vasculature.

genitors present (Jenkinson et al. 2005). The interdependence between thymocytes and TEC is stepwise. Cortical TEC but not medullary TEC are present in $RAG^{-/-}$ mice, which have a block at the DN2/3 stage (Klug et al. 1998; van Ewijk et al. 2000). SCID mice, which similarly are unable to generate T-cell receptor-expressing thymocytes, have small thymuses with disorganized structure, with only scattered mTEC; yet when TCR-transgenic thymocytes expressing a full TCR are present in SCID mice, a medulla develops (Shores et al. 1991; Shores et al. 1994). These interactions between thymocytes and TEC are not limited to the early postnatal period of greatest thymic productivity. TEC populations are not static, but rather are continuously differentiating and turning over (Gray et al. 2006). Thymocytes are constantly being renewed by an input of T-progenitors. The interdependence of thymocytes and TEC may contribute to their mutual decline with aging. Yet this same interdependence may underlie the ability of thymuses to renew growth and expand.

3 Thymic Involution

The thymus attains its greatest size and cellularity in the late fetal and early neonatal period. The overall physical size of the thymus remains relatively constant after early childhood (Steinmann et al. 1985), but perivascular spaces containing connective tissue expand and thymic epithelial spaces are reduced, until thymic medullary and cortical tissues are limited to small islands surrounded by adipose and fibrous tissue (Gruver et al. 2007; Shiraishi et al. 2003). Thymocyte depletion begins in the subcapsular area and then declines throughout the cortex (Brelinska 2003). Cortical TEC markers gradually decline. The relative levels of thymocytes and stromal cells are reduced proportionately. By computerized tomography the large thymic profile and radiodense parenchyma evident in children dwindles into a much smaller profile in adults and appears merely as diffuse strands after middle-age (Hakim et al. 2005; Mackall et al. 1995; Sfikakis et al. 2005).

Despite the quantitative reductions in cortical and medullary tissue, thymopoiesis at some level continues throughout life (Haynes BF et al. 2000; Jamieson et al. 1999; Naylor et al. 2005). The thymus continues to generate new T-cells into the adult years and even into old age (Douek et al. 2000; Jamieson et al. 1999; Nasi et al. 2006; Naylor et al. 2005). The naïve T-cells that are newly generated in aged mice appear functionally normal, capable of germinal center formation and support of humoral immunity (Haynes L et al. 2005). In adoptive transfer studies into irradiated aged host mice, thymuses seem capable of supporting positive and negative selection (Mackall et al. 1998). Thus the main effect of thymic involution is quantitative—concomitant with involution, the level of productive thymopoiesis declines. By 40–50 years of age the thymus is producing only about 10% its maximal capacity (Flores et al. 1999). Phenotypically naive T-cells ($CD45RA^{+}CD45RO^{-}CD62L^{+}CCR7^{+}CD95^{-}$) are present in the peripheral blood even in the elderly, but their numbers dwindle and fewer of these cells express markers of recent thymic emigrants (RTE)—CD31 in CD4+ T-cells and CD103 in CD8+ T-cells respectively

(Kimmig et al. 2002; McFarland et al. 2000; Nasi et al. 2006). This decline in naïve T-cells in the peripheral blood is paralleled by the decline in cells containing T-cell receptor rearrangement excision circles (TREC). TREC are non-replicating episomal DNA circles generated during V(D)J rearrangement in TCR β and α chain formation. The most commonly measured TREC, the signal joint TREC (sjTREC), is generated through the excision of the TCRδ locus during the rearrangement of the TCRα locus (Douek et al. 1998). Because most intrathymic expansion has been completed at this point, sjTREC are found in a high percentage of DP and SP thymocytes and in RTE in the peripheral blood. Because episomal DNA does not replicate, TREC frequencies are diluted by activation-induced or even homeostatic T-cell proliferation (Hazenberg et al. 2002). TREC frequencies in peripheral blood T-cells decline with age, reflecting both the decline in the level of thymopoiesis and the dilutional effects of T-cell proliferation (Douek et al. 1998; Gruver, Hudson and Sempowski 2007). Indeed the 2-log decline in TREC frequency between young adults and elderly is more extreme than the decline in phenotypically naïve T-cells; the numbers of phenotypically naïve cells are maintained by increased homeostatic cycling despite falling thymic production (Naylor et al. 2005; Wallace et al. 2004).

4 Capacity for Renewal of Thymopoiesis

Despite this gradual age-dependent decline in thymic productivity and structure, the adult thymus is remarkably capable of renewal of thymopoiesis after severe peripheral cytoreduction (Douek and Koup 2000; Hakim et al. 2005; Sfikakis et al. 2005). In a cohort of middle-aged to elderly patients undergoing autologous hematopoietic stem cell transplant for treatment of breast cancer, we were able to examine the frequency, timecourse and consequences of thymic recovery without the presence of confounding factors such as hematologic malignancy, immunosuppressive drugs or graft-versus-host disorder (Hakim et al. 2005).

We assessed thymic structural change during the post transplant period by evaluating serial thoracic CT scans using a 4 point thymic size index (Hakim et al. 2005; Kolte et al. 2002; McCune et al. 1998). The thymic profile was extremely reduced in size by the end of transplant conditioning (thymic index (TI) = 0) and in most patients thymic size remained minimal after transplant. In one third of the patients, however, thymic size gradually increased, attaining a maximum TI of at least 2, the size of the typical thymus in middle-aged adults (Hakim et al. 2005; McCune et al. 1998). Furthermore 7 of 32 patients achieved a TI of at least 3, a significantly larger thymic profile with moderate cellularity. This change in size and radiodensity is particularly remarkable in that only 2 of these patients had a TI of 3 prior to the start of therapy. Thus the development of a radiodense thymic profile post transplant in these patients represented not merely a return to the pretreatment status, but an increase over their previous status.

Two points are worth noting. The first is that the maximum thymic size attained correlated strongly with age. Whereas 4 out of 5 of the patients aged 30–39 showed

a significant thymic enlargement, the incidence of thymic recovery dropped to only 6 of 13 patients among those aged 40–49, and only 2 of 14 over 50 years of age demonstrated any thymic enlargement from the treatment nadir (Hakim et al. 2005). Second the development of thymic enlargement proceeded very slowly, requiring 6–12 months in younger patients to reach maximal size and as long as 24 months in older patients showing thymic recovery.

The changes in thymic profiles represented a renewal of thymopoiesis. The recovery of radiodense thymic mass correlated strongly with the recovery of newly matured $CD4^+$ T-cells in the peripheral blood. Because more than 95% of naive $CD4^+$ T-cells are lost during transplant regimens, the reappearance and increase of phenotypically naïve T-cells post transplant can provide an estimate of recovery of newly matured cells and hence an assessment of thymic function (Hakim et al. 2005; Mackall et al. 1995). Following autologous HSC transplant, levels of naive ($CD45RA^+CD62L^+$) $CD4^+$+ T-cells remained low, returning to normal levels of naïve cells only in the second year, even in patients with the best thymic recovery (Hakim et al. 2005). Consistent with the pattern of thymic enlargement, the levels of naïve cells at the end of 2 years—whether assessed by phenotypic markers or by quantitative PCR of TREC—were strongly age-dependent and correlated with the maximum thymic expansion. Finally, a broad TCR repertoire diversity appeared within $CD45RA^+$ naïve $CD4^+$ T-cells within a few months after transplant. Hence the thymic role of generating broad TCR repertoire diversity was maintained in the restored thymus post transplant (Hakim et al. 2005).

In HIV seropositive patients, initiation of highly-active antiretroviral therapy (HAART) has similarly resulted (after a several month lag) in increased thymic volume and cellularity, and enhanced metabolic activity as assessed by PET imaging (Hardy et al. 2004; Hudson et al. 2007). Rapid early increases in CD4 numbers have occurred after HAART, but these were due to trafficking, increased T-cell survival and peripheral expansion (Bucy et al. 1999; Pakker et al. 1998). In contrast, the slow long-term increases in the total CD4 count after HAART were accompanied by increases in the numbers of naïve CD4 and sjTREC in the peripheral blood, indicative of a renewal of functional thymopoiesis (Dion et al. 2004; Dion et al. 2007; Douek et al. 1998; Hudson et al. 2007). As in the studies of transplant patients, however, the recovery of naïve populations required months to appear and the frequency of successful renewal of thymopoiesis declined with age (Dion et al. 2004; Dion et al. 2007; Hudson et al. 2007).

5 Control Points of Thymic Involution and Renewal

The capacity of the adult or even the aged thymus to expand and increase both thymocyte and TEC content is not limited to rebound from transplant or HIV infection. When aged porcine thymic lobes were placed in young swine as vascularized renal grafts, the thymuses were rejuvenated. Expanded TEC and thymocyte populations appeared, and became organized into densely cellular cortical and medullary

structures (Nobori et al. 2006). Infusion of normal hematopoietic stem cells into IL-7R$\alpha^{-/-}$ mice resulted in not only an influx of normal thymocytes into the stunted thymus, but a marked increase in thymic size and cellularity (Prockop et al. 2004). Treatment with a variety of cytokines and systemic hormones has been found to enhance thymic recovery after transplant and to renew thymic size and productivity even in aged hosts, as described in section-6. Thus the adult thymus shows a remarkable ability to reverse involution, to increase thymic epithelial space and enhance productive thymopoiesis. Given this plasticity, therefore, it is important to identify the elements which control thymic size and productivity in involution and in renewal. The conditions of cytoreductive transplant regimens and the addition of various agents can impact the thymopoietic process at multiple levels. Current research points to 3 main control points determining the status of thymopoieisis: the number of functional T-progenitors that migrate to the thymus, the number of available "niches" for such cells to enter and initiate thymopoiesis, and the productive capacity of the thymopoietic maturational process itself (see Fig. 1).

5.1 Stem Cells

The capacity to generate T-cells is ultimately dependent upon the availability of functional T-progenitors. When marrow from normal mice was mixed with that from mice with T-cell maturational blocks, the final output of T-cells was directly dependent on the proportion of competent progenitors (Almeida et al. 2001). Conversely, when T-progenitors isolated from marrow or generated ex vivo by Notch signaling were infused into irradiated mice, the increased T-progenitor doses enhanced thymocyte numbers, TREC and peripheral T repopulation (Chen et al. 2004; Zakrzewski et al. 2006). Increasing evidence suggests that age-dependent declines in the levels of marrow-derived T-progenitors are a key element in decreased thymic productivity. When equivalent numbers of T-depleted marrow from young and old mice have been transplanted into irradiated young hosts, the aged marrow generated fewer peripheral T-cells (Mackall et al. 1998). Competitive thymic repopulation studies using mixtures of young and aged marrow have further determined that the aged marrow gave rise to only one tenth as many DP-thymocytes as the young marrow (Zediak, Maillard and Bhandoola 2007). The problem was not an engraftment failure; the aged marrow-derived progenitors were less productive even when injected directly into the thymus, or when cultured with Notch-ligand expressing stroma ex vivo (Zediak, Maillard and Bhandoola 2007).

These adoptive transfer and culture studies point to quantitative and qualitative changes in the marrow derived T-progenitor population. There is no evidence of a quantitative deficit in marrow of the long-term self-renewing HSC (LT-HSC). Earlier studies suggesting that LT-HSC increased 5 fold with age have been substantiated with current multiparameter cytometry (Rossi DJ et al. 2005; Rossi DJ et al. 2007; Sudo et al. 2000). The LT-HSC, which are mostly quiescent, give rise to MPP, that in turn become committed to myeloid and lymphoid lineages in response to ligand-

receptor interactions and growth factors provided by the marrow microenvironment. In adoptive transfer experiments, the MPP in aged mice retained the capacity to generate myeloid populations, but lymphoid lineages were markedly reduced compared to MPP from young mice (Rossi DJ et al. 2005; Zediak, Maillard and Bhandoola 2007). It is this skewing away from lymphoid commitment that has been proposed to underlie declining lymphoid progenitor activity.

One unresolved question is whether the changes in lymphoid progenitors are due to intrinsic changes in the stem cells or to age-dependent changes in the marrow microenvironment. Rossi has argued persuasively for intrinsic changes. He determined that LT-HSC expressed a broad diversity of genes believed to be restricted to more mature and lineage-committed cell types, suggesting that transcription of lineage associated genes in stem cells occurred prior to full lineage commitment, if not as a requirement of that differentiation. When lineage-associated genes were compared in young and old HSC, marked changes in gene expression were observed, consistent with a pattern of reduced commitment toward lymphocytes and increased commitment to myeloid lineage. Genes consistent with lymphoid development such as IL-7R and Flt3 were reduced while myeloid genes were increased (Rossi DJ et al. 2005; Rossi DJ, Bryder and Weissman 2007). Since these changes occurred in the LT-HSC, preceding lineage commitment, these data support an intrinsic model of HSC decline. On the other hand, the marrow compartment undergoes aging-dependent changes that may well impact on stem cells. The most primitive long term HSC are maintained in the marrow in the calcium rich environment along the bone. In these endosteal niches, osteoblast cells producing osteopontin regulate HSC proliferation (Haylock et al. 2006). Osteoblasts provide growth factors and express Notch ligands such as Jagged-1 that shape HSC expansion and differentiation (Calvi et al. 2003; Weber et al. 2006). Administration of osteoblasts or bone fragments at the time of transplant has enhanced HSC engraftment (El-Badri et al. 1998). Furthermore administration of parathyroid hormone, which stimulates osteoblast growth, markedly increased the number of stem cells in intact mice and improved survival after transplant with limited HSC doses (Calvi et al. 2003). Finally, purified primary murine osteoblasts, cultured with parathyroid hormone, supported the full differentiation of HSC into mature B-cells whereas cytokines produced by nonosteoblast stroma shifted the cultures instead toward myeloid differentiation (Zhu et al. 2007). These last data would support a microenvironment model, one that suggests that declines in osteoblast and calcium-rich bone levels in the elderly skew the marrow microenvironment toward stromal elements favoring myeloid commitment.

Whether T-progenitor changes are intrinsic or marrow microenvironment-induced, these models would propose that a decline in committed T-progenitors would gradually starve the thymus of new progenitors and, in the absence of adequate numbers of developing thymocytes, the TEC would decline. Min et al. have determined that while thymic DN1 levels appeared to remain constant, the number of thymic ETP in unmanipulated aged mice was reduced 40-fold as compared to those in young mice (Min H et al. 2004). A gradual age-dependent decline in T-progenitors could therefore contribute to thymic decline.

5.2 Changes in Thymic Niches

The second control point for thymopoiesis is the entry or engraftment of T-progenitors into the thymus. The ability of progenitors to productively engraft is constrained by thymic elements. Much of the evidence for this is indirect. Entry of progenitors into the thymus is not a continuous process but rather a gated event; progenitor entry occurs in waves during embryogenesis and in adulthood, at least in mice, with a periodicity of 3–5 weeks in nonirradiated mice (Goldschneider 2006). Adoptive transplant experiments have shown that the number of progenitor binding sites in the thymus is limited and can be saturated (Foss et al. 2001). Treatments such as KGF (see below) can increase the number of engraftment sites, as measured by uptake of labeled progenitors (Rossi SW et al. 2007). Furthermore, functional and dysfunctional DN thymocytes can compete for these limited numbers of sites (Prockop and Petrie 2004). Capacity to productively mature into T-cells does not determine occupancy of progenitor niches; occupancy by $Rag^{-/-}$ thymocytes can block engraftment of normal progenitors (Prockop and Petrie 2004). This may be particularly relevant to aging given Min's findings that ETP in aged mice were not only severely reduced in number, but that these cells were less functional than ETP from young mice (Min H, Montecino-Rodriguez and Dorshkind 2004). If dysfunctional ETP occupying thymic niches accumulate (since they do not mature and "move on"), then productive thymopoiesis could be progressively reduced. This is an intriguing hypothesis, in that the ablation of dysfunctional (as well as functional) ETP by transplant irradiation or chemotherapy regimens could open up these niches for new engraftment. Such a general clearance of niches could contribute to the thymic renewal and expansion observed after transplant in man (Hakim et al. 2005).

The mechanisms determining progenitor engraftment "niches" remain unknown. Part may relate to expression (on progenitors or thymus) of the factors regulating T-progenitor homing. In the fetus thymus, T-progenitors depend on chemotaxis to migrate into the thymic anlaga. CC-chemokine ligands 21 (CCL21) and CCL25 on TEC interact with their corresponding receptors CCR7 and CCR9 on progenitors (Takahama 2006). Later, ETP enter through the vasculature at the CMJ (Lind et al. 2001). Although CCR9 deficiency reduces homing, it is unclear whether chemokines are specifically involved in homing or in drawing engrafted DN1 cells away from the CMJ (see 5.3 below) (Petrie et al. 2007; Takahama 2006). In contrast, the interaction of P-selectin on thymic endothelium and P-selectin ligand (PSL1) on circulating thymic progenitors plays a significant role in uptake through the CMJ vasculature (Rossi FM et al. 2005). Furthermore, the number of thymic progenitors present in the inner cortex can affect expression of P-selectin on the endothelial cells, a negative feedback loop which may play a role in gating entry of progenitors (Rossi FM et al. 2005).

5.3 Changes in Productive Expansion of DN Thymocytes

Thymic productivity is determined not only by progenitor engraftment in the thymus, but by the proliferative expansion of DN thymocytes, the process by which small numbers of progenitors can increase many thousand fold. Assessment of TREC provides evidence that the degree of expansion of DN cells during the process of thymic maturation declines with age. Although the overall number of thymocytes declines with age, the ratio of sjTREC per 10^5 thymocytes remains constant (Jamieson et al. 1999; Sempowski et al. 2000). This does not necessarily mean that increasing the supply of progenitors would increase thymopoiesis in a straightforward manner. The vast majority of thymocytes are DP cells that have just completed TCRα chain rearrangement and therefore most of these cells contain sjTREC. Dion has further analyzed thymic productivity by measuring the ratio of sjTREC (generated at the end of DN4 thymocyte proliferative expansion) to DβJβTREC (generated early in the TCR β-chain rearrangement process) (Dion et al. 2004). This ratio therefore measures the extent of proliferative expansion occurring during the main DN3/DN4 period of thymocyte increase. This ratio steadily declines with age (Dion et al. 2004). Dion's analysis of TREC ratios was particularly informative in the renewal of thymopoiesis with HAART therapy. The ratio of sjTREC to DβJβTREC increased after HAART therapy indicating an increase in the proliferative expansion of DN thymocytes during maturation, that is, an increase in thymic productivity (Dion et al. 2004; Dion et al. 2007).Thus the structural changes in the thymus are associated with a lower intrathymic proliferative expansion of progenitors, resulting in a lower thymic productivity.

The mechanisms regulating the extent of DN expansion are not fully resolved, but it is well supported that this process involves the close association of DN thymocytes with cortical TEC cells and the factors they produce during the DN migration from the CMJ vasculature outward to the SCZ of the cortex. T-cell commitment occurs in DN1 and DN2 thymocytes by recurrent signaling through Notch by its Delta-like-1 ligand on TEC-cells (Schmitt et al. 2004). TEC-cells produce IL-7, which provides a necessary survival signal during the DN1 transition to DN2 (Andrew et al. 2001; von Freeden-Jeffry et al. 1997). TEC-cells also produce the chemokines CCL21 and CCL25 that draw DN from the inner to the outer cortex and into the SCZ. Migration requires not only polarizing signals but a substrate for cell adhesion; TEC also produce the V-CAM1 that binds with the α4β1 integrins on DN-cells. The main expansion of DN occurs during this outward migration and in the SCZ, all in close association with TEC. It is recently been recognized that the TEC populations are not static but rather are maintained in a dynamic equilibrium with thymocytes (Gray et al. 2006). The wave of proliferative expansion of TEC produced by factors like keratinocyte growth factor (KGF), are immediately followed by a wave of expansion of thymocytes (Rossi SW et al. 2007). Thus factors that stimulate TEC expansion can result in expansion of thymocytes.

6 Factors Regulating Thymic Involution and Supporting Thymic Renewal

Over the last decade several factors have been identified that can effectively act on one or more of the control points in thymopoiesis (see Fig. 1). These factors can be broadly subdivided into those produced systemically in the body, those generated by the thymic stromal cells, and finally those intrinisic to the hematopoietic-lineage thymocytes themselves. Some of these factors may interact with thymopoiesis at multiple levels. Nevertheless examination of these 3 categories is useful in terms of suggesting potential avenues for thymic renewal.

6.1 Systemic Hormones

Thymopoiesis can be significantly affected by systemic hormones; age-related changes in these may therefore contribute to involution or support renewal. One candidate is the growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis. Pituitary growth hormone (GH) levels peak in man early in the third decade of life and decline with age. Most of the actions of GH are carried out by IGF-1, which is generated in the liver in response to GH, but is also produced by TEC (de Mello Coelho et al. 2002). Preclinical studies in aged mice as well as studies in lymphopenic HIV^+ patients have consistently found that treatment with either GH or IGF-1 can produce an increase in thymic cellularity and circulating naïve T-cell levels (Montecino-Rodriguez et al. 1998; Napolitano et al. 2002). Administration of IGF-1 or GH accelerates enhances hematopoietic and immune reconstitution after hematopoietic stem cell transplant in murine transplant models (Alpdogan et al. 2003; Chen et al. 2003). GH and IGF-1 may affect thymopoiesis at two levels. IGF-1 treatment increases lymphoid progenitors in the marrow, resulting in increases in pre and pro B-cells as well as increasing the supply of functional DN thymocytes (Alpdogan et al. 2003). IGF-1 also increases production of extracellular matrix by TEC and increases thymocyte adhesion to TEC (de Mello Coelho et al. 2002). Since the earliest T-progenitors migrate from the vasculature at the cortico-medullary junction to the outer subcapsular epithelium in the course of their proliferative expansion, factors that enhance DN interactions with TEC and accelerate this migration could enhance thymic productivity. Yet the level of GH is not the main determinant of involution. Thymic size is normal and involution rate is not significantly different in GH-deficient Little (*lit/lit*) mice and their normal littermates (Min H et al. 2006). Furthermore, while GH treatment can produce a doubling in thymic cellularity in old mice, just as in young ones, this increase does not reverse the much greater decline accompanying age-dependent involution (Montecino-Rodriquez et al. 2005).

While the declining levels of GH and IGF-1 may reduce lymphopoiesis, it is the converse, the post-pubertal rise in gonadal steroids—androgens, estrogens and progesterone—that may contribute to the involutional process. Gonadal steroid treatment induces involutional changes in the thymus, whereas castration or ovariectomy in rodents results in thymic enlargement, and increased thymic and peripheral T-cell populations, even in aged animals (Greenstein et al. 1986; Leposavic et al. 2001; Windmill et al. 1998). The effects of androgens on thymopoiesis are mediated through TEC, as demonstrated by experiments involving reciprocal marrow transplants between normal mice and those lacking expression of androgen receptors (Olsen et al. 2001). Drugs blocking testosterone production are equally as effective as surgical treatment. Treatment of aged rats with luteinizing hormone-releasing hormone (LHRH) analogue produced a significant increase in thymic weight (Kendall et al. 1990). Following autologous or allogeneic hematopoietic stem cell transplant, treatment with an LHRH agonist enhanced thymic recovery and increased the numbers of circulating naïve $CD4^{+}$+ T-cells (Goldberg et al. 2005; Goldberg et al. 2007; Heng et al. 2005; van den Brink et al. 2004). These data support the role of systemic levels of gonadal hormones in modulating thymopoiesis. It must be remembered however that progressive thymic decline in man begins in the first year of life, not at puberty. Hypogonadal mice do not have delayed thymic involution (Min H, Montecino-Rodriguez and Dorshkind 2006). Additional mechanisms must therefore contribute to thymic involution.

6.2 TEC Generated Cytokines—IL-7

Because of the critical role of TEC in all aspects of thymopoiesis, changes in TEC could regulate thymopoiesis. One mechanism proposed for thymic involution is a decline in TEC production of the cytokine IL-7, which is necessary for thymocyte maturation from DN1 to DN2. T-cell maturation was severely reduced in both IL-7 and IL-$7Ra^{-/-}$ mice(Peschon et al. 1994). In mice (although not in man), the level of IL-7 mRNA declined with age (Andrew et al. 2002). IL-7 therapy in vivo and in vitro reduced the apoptotic loss of thymocytes during the DN1->DN2 transition in aged mice (Andrew and Aspinall 2002; Phillips et al. 2004). Systemic IL-7 treatment also sped recovery of thymopoiesis following marrow transplant into irradiated hosts (Alpdogan et al. 2001; Bolotin et al. 1996). But IL-7 effects on thymopoiesis seemed to be greatest under conditions of TEC damage. IL-7 supplementation post transplant may have been replacing cytokine production lost by radiation damage to stromal cells (Chung et al. 2001). Supplemental IL-7 therapy has had only limited effects on thymopoiesis in intact hosts. IL-7 treatment did not increase thymic size or productivity in young mice (Chu et al. 2004), and short term IL-7 treatment in aged mice produced no increase in overall thymopoiesis (Sempowski et al. 2002). Marrow stroma also produce IL-7, which plays a significant role in early B-lymphoid development. Addition of IL-7 to IGF therapy had additive effects on marrow B-cell development, but did not further enhance

thymopoiesis (Alpdogan et al. 2003). The strongest evidence of the limitations of IL-7 in contolling thymopoiesis come from studies of long-term IL-7 augmentation by injection of IL-7 producing stromal cells into the thymus in young mice (Phillips et al. 2004). When these mice with elevated intrathymic IL-7 production were monitored for up to 2 years, the levels of DN1 thymocytes transiting to the DN2 stage were maintained in aged mice, but structural involution of the thymus and the age-dependent decline in DP and SP thymocytes were not altered. The age-dependent decline in DN4 proliferative expansion continued unchanged despite elevated IL-7 (Phillips et al. 2004). Thus the decline in thymopoietic productivity is not dependent primarily on TEC IL-7 production.

6.3 Thymocyte Generated Cytokines—KGF

Keratinocyte growth factor, also known as fibroblast growth factor 7 (FGF-7), is produced in the mature thymus by DP- and SP-thymocytes (Erickson et al. 2002; Jenkinson et al. 2003). The TEC express the receptor (FGFR2IIIb), which binds KGF as well as the mesenchymally derived FGF-10 (Min D et al. 2002; Rossi SW et al. 2007). Unlike FGF-10. KGF is not necessary for initial thymic organogenesis, but plays an important role in the adult in renewing thymopoiesis post cytoreduction (Alpdogan et al. 2005). KGF treatment increased the uptake of labeled T-progenitors, that is the number of engraftment niches (Rossi SW et al. 2007). KGF treatment in adult mice also stimulated growth of TEC-precursors and expansion of TEC, resulting shortly afterwards in a wave of proliferative expansion in DN-thymocytes (Rossi SW et al. 2007). In aged mice or in *klotho* mice, an aging model showing early thymic involution, KGF treatment increased thymopoietic capacity and reversed involutional changes (Min D et al. 2007). Repeated monthly KGF treatments prolonged these effects and reversed involution in aged murine thymic structure, returning the thymuses to the size of those in young adults (Min D et al. 2007). The KGF results also point up the interconnections between thymocytes and TEC. $RAG^{-/-}$ thymocytes, perhaps because they are arrested prior to the DP stage, do not produce KGF (Erickson et al. 2002). The $RAG^{-/-}$ medullary region is rudimentary and disorganized in mice, but can be induced to develop either by transplant of normal hematopoietic stem cells (van Ewijk et al. 2000), or by treatment with KGF (Erickson et al. 2002).

Thus factors such as systemic hormonal shifts and intrinsic cytokine programs within thymocytes and TEC-cells can all affect thymopoiesis. The complex interactive web linking thymocyte and TEC survival and differentiation acts as an amplifying factor. Increasing the input of functional thymic progenitors can trigger an expansion of TEC, which create in turn new niches for T-cell lineage commitment and supports increased thymocyte proliferation. Alternatively, in aging, the decline in these factors may reinforce a downward spiral resulting in thymic involution.

7 Conclusions

Aging is associated with a progressive decline in the generation of new T-lymphocytes, with consequent losses in repertoire diversity and functional competence. The age-dependent involution of the thymus underlies this loss. Achieving its greatest size in the neonatal period, the thymus undergoes a steady lifelong decline in structure and productive thymopoiesis. Yet the presence of thymic renewal in adults—following autologous transplantation in cancer patients or HAART therapy in HIV$^+$+ individuals—demonstrates that the thymus is capable of regrowth. Multiple experiments in animal models have demonstrated dramatic increases in thymic size and productivity. Thus the decline in thymopoiesis is not irreversible.

Our understanding of the regulation of thymic structure and thymopoietic productivity is in a rapid state of flux. The availability of recombinant cytokines and transgenic and knockout mice have shaped our concepts of the cellular and cytokine factors regulating lymphocyte generation and homeostasis. Thymopoiesis is dependent upon a continuing supply of T-lymphoid progenitors, maintenance of open thymic niches for progenitor engraftment and support of DN migration and productive expansion by the cortical stromal microenvironment. All of these are regulated by reciprocal interactions between the marrow and thymic stromal elements and developing lymphocytes, involving both cytokine/chemokine signals and direct cell contact mediated signalling. Novel strategies have been tested to enhance progenitor numbers by supporting osteoblast growth (Ballen et al. 2007; Calvi et al. 2003; Zhu et al. 2007), or to directly stimulate early lymphoid progenitors with IGF or IL-7 (Alpdogan et al. 2003), or to bypass the marrow completely and expand committed T-progenitors ex vivo (Zakrzewski et al. 2006). On the thymic stromal side, factors such as IGF, KGF or LHRH agonists have produced increases in TEC and subsequent increases in productive thymopoiesis. Combinations of these therapies may provide the means to reverse thymic decline and renew the generation of naïve T-cells in adults or even in the aged. Although many questions remain, such treatments might provide a long-term benefit in reversing immunosenescence.

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Inflammation

Inflamm-Aging

L. Bucci, R. Ostan, M. Capri, S. Salvioli, E. Cevenini, L. Celani, D. Monti and C. Franceschi

Contents

1 Introduction

The function of immune system depends on a subtle and well tuned network of humoral mediators, collectively called cytokines, responsible for differentiation, proliferation and survival of lymphoid cells. They include colony stimulating factors, and cytokines such as interferons and tumor necrosis factors (TNFs). These molecules constitute a complex network: cytokines, such as IL-2, have a particular importance for the proliferation and differentiation of T, B, and NK cells. IL-2 and IL-10 lead to an increased production of IgM, IgG and IgA, whereas IL-4 and IL-13 induce IgE and IgG4 synthesis. Other cytokines, such as IL-1, IL-6 and TNF-α are considered proinflammatory agents, and play an important role in the immune response and inflammation.

C. Franceschi (✉) · L. Bucci · M. Capri · S. Salvioli
Department of Experimental Pathology
University of Bologna, Via San Giacomo 12
I-40126 Bologna, Italy
Tel.: +39 051 209 4743
Fax: +39 051 209 4747
E-mail: claudio.franceschi@unibo.it

R. Ostan · D. Monti
Department of Experimental Pathology and Oncology
University of Florence, Viale Morgagni 50
I-50134 Florence, Italy

M. Capri · S. Salvioli · E. Cevenini · L. Celani · C. Franceschi
CIG-Interdepartmental Center "L. Galvani"
University of Bologna, Via San Giacomo 12
I-40126 Bologna, Italy

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It's widely accepted that many of the most important age-associated diseases, such as cardiovascular diseases, atherosclerosis, Alzheimer's disease, arthrosis and arthritis, sarcopenia and diabetes share a common inflammatory background (Appay and Rowland-Jones 2002; Boren and Gershwin 2004; Cappola et al. 2003; Licastro et al. 2003; Roubenoff et al. 2003a,b; Szmitko et al. 2003; Zanni et al. 2003). Inflammatory reactions are a complex series of physiological events designed to limit insult and promote repair. During aging it has been observed a complex remodelling of the immune system responsible for a series of age-related phenomena, among which a profound modification within the cytokine network. The typical feature of this phenomenon is a general increase in plasmatic levels and cell capability to produce proinflammatory cytokines. The first evidence of this age-associated modification in the balance of cytokine network was described by Fagiolo et al. (1993) who found an increase of IL-6 plasma levels and a decrease of IL-2 production in healthy elderly subjects (Fagiolo et al. 1993; Franceschi et al. 1995). Moreover, the authors described a significant increase of IL-6, TNF-α and IL-1β levels in mitogen-stimulated cultures from aged donors. These data indicated that the cellular machinery for the production of these cytokines is well preserved in aging, and also that cells from old people are able to up-regulate their production in response to appropriate stimuli. The well established increase with age of IL-1, IL-6 and TNF-α plasma levels appears to be unexpectedly present either in persons who enjoyed successful aging and those who suffered age-associated pathologies. This increase continues with age, until the extreme limit of human life, and high levels of IL-6 are found in healthy centenarians (Baggio et al. 1998). In these exceptional individuals other inflammatory factors, such as acute phase proteins, lipoprotein a [Lp(a)], fibrinogen, coagulation factors, and other proinflammatory cytokines are similarly increased (Baggio et al. 1998; Bruunsgaard et al. 1999; Mannucci et al. 1997; Mari et al. 1995). Thus, even if high levels of IL-6 have been indicated as one of the most powerful predictors of morbidity and mortality in the elderly (Ferrucci et al. 1999; Harris et al. 1999), an inflammatory status is compatible with extreme longevity and paradoxically proinflammatory condition have been documented in centenarians in relatively good health (category A and B as in Franceschi et al. 2000a). Another proinflammatory cytokine, IL-18, increases with age and centenarians display significant higher serum levels compared to people of younger ages. However, higher levels of IL-18-binding protein, a protein which binds and neutralizes IL-18, is also increased, suggesting that compensatory mechanisms capable of quenching the proinflammatory activity of IL-18 likely occur with age (Gangemi et al. 2003). In addition, the reshaping of the cytokine network in aging is extended to chemokines and proinflammatory molecules regulating monocyte and T lymphocyte recruitment towards sites of inflammation. The production of chemokines such as RANTES, MIP-1α, IL-8 and MCP-1 is increased in the elderly with clear consequences for the inflammatory mechanisms and the recirculation of lymphocyte subsets (Gerli et al. 2000; Mariani et al. 2002).

This chronic, low grade, proinflammatory condition was named *inflamm-aging* (Franceschi et al. 2000b,c) and it is characterized by a general increase in the

production of inflammatory cytokines and a subsequent rise of the main inflammatory markers, such as C-reactive protein (CRP) and serum amyloid A. It is at the present unknown whether the derangement in the regulation of inflammatory reactions is a cause or rather an effect of the aging process as a whole. Nevertheless, an altered inflammatory response can probably be the result of the chronic exposure to stressors, such as antigens, leading to a progressive activation of macrophages and related cells in most organs and tissues of the body, but also to chemical and physical agents that threaten the integrity of the organism (Franceschi et al. 2000b). The chronic proinflammatory status can be in some cases an important cause of damage, by itself or by interacting with other pathological molecular mechanisms, thus contributing to the acceleration of the onset of different diseases or their severity. Indeed, it has been demonstrated that a proinflammatory status is related to mortality risk for all causes in older persons (Bruunsgaard et al. 2001) rendering the subjects more prone to a variety of infectious and noninfectious diseases (cardiovascular diseases, neurodegenerative disorders, osteoporosis, sarcopenia and diabetes, among others; De Martinis et al. 2005).

2 Memory Cells and Filling of Immunological Space

Immunosenescence is not accompanied by an unavoidable and progressive deterioration of the immune function, but is rather the result of a remodelling where some functions are reduced, others remain unchanged or even increased. Both humoral and cell-mediated specific immune response are modified and remodelled by aging. The ancestral/innate compartment of the immune system appears relatively preserved during aging in comparison to the more recent and sophisticated adaptive compartment that exhibit more profound modifications. Clinical evidence indicates that with advancing age, immune responses against recall antigens may still be conserved (Ahmed et al. 1996), but the ability to mount primary immune responses against novel antigens declines significantly (Weigle 1989). The impaired ability to mount immune responses to new antigens may result in an higher susceptibility to infectious diseases and may limit the efficacy of vaccination strategies in elderly people.

In fact, one of the main characteristics of immunosenescence is the process termed *thymic involution*, responsible for a progressive, age-related reduction in size of the thymus, due to profound changes in its anatomy, associated with loss of thymic epithelial cells and a decrease in thymopoiesis. This decline in the output of newly developed T-cells results in a diminished number of circulating naïve T-cells and an impaired cell mediated immunity (Fagnoni et al. 2000). A major consequence of thymic involution is a profound age-related change in T lymphocyte subpopulations (Nasi et al. 2006).

The rate of naïve T-cell output from the thymus dramatically declines, and memory T-cells proliferate in the periphery to replace the loss of thymic output, a phenomenon called *homeostatic expansion* (or *proliferation*; Aspinall et al. 2000, Berzins et al. 2002).

Thus, the loss of naïve T-cells, able to cope with new antigens, leads to the accumulation of memory and effector cells, a phenomenon described as "filling of the immunological space" (Franceschi et al. 2000b,c; Luciani et al. 2001). Indeed, we demostrated that aging is accompanied by an increase of memory T-cells, and this phenomenon is different in $CD4^+$ and $CD8^+$ T-cells (Cossarizza et al. 1996). The concomitant occurrence of these two phenomena, i.e., decrease of virgin T-cells and increase of memory T-cells, related to thymic involution and lifelong antigenic load, respectively, is the most important characteristics of immunosenescence and of its clinical correlates.

The exhaustion of thymic output occurring during aging is also confirmed by phenotypic analysis, and this phenomenon is more rapid and evident in $CD8^+$ T-cells (Fagnoni et al. 1996, 2000; Franceschi et al. 1995; Zanni et al. 2003). Recently, $CD31^-CD4^+$ T-cells were identified as an autonomously regulated subset, characterized by a highly restricted oligoclonal TCR repertoire, which constitutes a pool of naïve T-cells not affected by thymic decline, likely playing a central role in adaptive immunity and providing sufficient number of naïve $CD4^+$ T-cells in the elderly, even in the presence of a drastically reduced thymic function (Kohler et al. 2005). T-cells accumulating with age are mainly $CD28^-$ T lymphocytes in both $CD8^+$ and $CD4^+$ subsets (Fagnoni et al. 1996; Valenzuela et al. 2002; Zhang et al. 2002). CD28 serves both as a costimulatory molecule for T-cell activation (Krause et al. 1998; Sepulveda et al. 1999) and as a signal for glucose transport (Frauwirth et al. 2002). $CD28^-$ T-cells display several aspects of senescence, including oligoclonal expansion (Batliwalla et al. 1996), shortened telomeres (Effros 1997; Valenzuela et al. 2002), limited proliferative potential (Effros 1997; Valenzuela et al. 2002, Vallejo et al. 2001), production of TNF-α and IL-6 (Zanni et al. 2003), and resistance to apoptosis (Brzezinska et al. 2004; Posnett et al. 1999). Many studies indicate that the memory pool is composed of different subsets based on the expression of chemokine receptors, selectins, and costimulatory receptors. *Central memory T-cells* (TCM) bear lymph node homing receptors (L-selectin, CD62L, and CC-chemokine receptor 7 [CCR7]) and costimulatory molecules, such as CD27 and CD28. These cells show a scarce effector function, but can have extensive replicative response to their specific antigen (Maus et al. 2004). *Effector memory* T-cells (TEM) have the capability to exert immediate effector functions (cytokines secretion and/or cytotoxic activity) and are characterized by the lack of CCR7 and by a heterogeneous expression of CD62L. Both the mentioned cell subsets have down-regulated the CD45RA, a marker of virgin T lymphocytes. Moreover, *terminally differentiated T-cells* (TTD), characterized by the expression of CD45RA (as naïve cells), the lack of CCR7 and CD62L, and usually of $CD28^-$, accumulate with age, particularly in $CD8^+$ T-cells (Pawelec et al. 2005). These profound age-related changes at the cellular level are accompanied by the peculiar, chronic, low grade proinflammatory status (inflamm-aging) suggesting that immunosenescence is mainly driven by a chronic antigenic load which not only induces an enormous expansion of $CD28^-$ T-cells, but also increases their functional activity, confirmed by an high frequency of cells positive for proinflammatory cytokines.

Indeed, a general trend towards an increase of both type 1 and type 2 cytokine-positive cells in naïve, memory and effector/cytotoxic CD8 T-cells was found. The increase of type 1 intracellular cytokines is particularly marked in memory and effector T $CD8^+$ lymphocytes. In old subjects, IFN-γ and TNF-α producing cells account for more than 60% of the $CD8^+$ T-cells. The increase of type 2 cytokines producing cells is lower when compared to type 1 and it results more evident in $CD8^+$ memory cells (Zanni et al. 2003).

The increased proinflammatory cytokines can be regarded as a double edged sword that at one side could be beneficial and protective in amplifying, via IFN-γ, the immune response against internal or external pathogens (Guidotti et al. 1996), and, on the other side, could be detrimental, later in life, via an excessive TNF-α and IFN-γ production capable of sustaining chronic inflammatory or autoimmune processes (Feldmann et al. 1997) that negatively correlate with human longevity.

Within this scenario, we can surmise that the continuous attrition caused by clinical and subclinical infections, as well as the continuous exposure to other types of antigens (food, allergens), is likely responsible for the chronic immune system activation and inflammation (De Martinis et al. 2004; Franceschi et al. 1999).

Emerging data suggest a possible contribution of CMV infection to this progressive, systemic, low grade proinflammatory status characteristic of immunosenescence. The age-dependent expansion of $CD8^+CD28^-$ T-cells, mostly positive for proinflammatory cytokines and including the majority of Cytomegalovirus (CMV)-epitope-specific cells, underlines the importance of chronic antigenic stimulation in the pathogenesis of the main immunological alterations of aging and may favor the appearance of several inflammatory pathologies (arteriosclerosis, dementia, osteoporosis, cancer; Sansoni et al. 2008).

Large clonal expansion of peripheral $CD8^+$ T-cells carrying receptors for single epitopes of CMV and Epstein-Barr Virus, detected using tetramer technology, are common in the elderly and are associated with a loss of effector memory cells, an increase of terminally differentiated $CD8^+$ cells and a gradual reduction of the immunological space (Franceschi et al. 2000c).

Functional T-cell responses to pp65 and IE-1 peptides, two CMV immunogenic proteins, performed on humans of different ages indicate that the pp65 is the major antigen against which aged people target their T-cells effector function with massive production of Th1 cytokines and increased presence of potential cytotoxic cells exhibiting degranulation markers (CD107a). Indeed, both CMV antigens are able to increase the production of IFN-γ and TNF-α in old subject in comparison with younger even if the CD4 and CD8 T- responses are not so similar. In fact, these two lymphocyte subsets respond differently to the same antigen and an inverse correlation exists between anti pp65-$INF\text{-}\gamma^+$ $CD4^+$ and $CD8^+$ T-cells (Vescovini et al. 2007).

On the whole, the existing literature suggests that CMV could represent one of the most important agent of effector T-cell expansion and a possible main mechanism underlying the persistent activation of the immune system in the elderly. This stable load of effector helper and cytotoxic T-cells producing IFN-γ and TNF-α

and having a potential cytolytic activity may be necessary to protect elderly people from CMV endogenous reactivation but, at the same time, may also became detrimental at the systemic and tissue levels. Finally, we can say that the expansion of functional effector T-cell producing high amounts of inflammatory cytokines may be considered as a general age-related phenomenon in CMV seropositive donors, that might give a substantial contribution to inflamm-aging (Vescovini et al. 2007).

Indeed, the number of functional CMV-specific CD8 cells is quite similar in young and old individuals. This is consistent with suggestion that these cells may contribute to the proinflammatory status often observed in the elderly and may contribute to frailty and mortality. Furthermore, in the elderly there is an accumulation of CMV-specific CD8 cells negative for CD28 and positive for the KLRG-1 and CD57. The presence of these two markers identifies dysfunctional CD8 T-cells that were not able to proliferate (Koch et al. 2007). In CMV seropositive individuals an accumulation of CMV-specific CD4 cells during aging is present. These cells are characterized by an effector phenotype ($CD28^-$, IFN-γ^+ and IL-2; Pourgheysari et al. 2007).

Moreover, the production of type 1 or type 2 cytokines by $CD4^+$ T-cells appears to be differently affected by aging process. Precisely, the percentage of INF-γ^+ cells decreases in virgin $CD4^+$ and in activated/memory T-cells from aged subjects in comparison with young subjects. The percentage of TNF-α^+ cells increases in activated/memory $CD4^+$ T subsets from nonagenarians. Concerning type 2 cytokines, IL-4^+ cells increased in activated/memory $CD4^+$ subset from nonagenarians suggesting a shift towards type 2 cytokines (Alberti et al. 2006).

3 Shrinkage of T-Cell Repertoire

Both quantitative and qualitative changes of T lymphocyte subsets are implicated in the age-related remodelling of the immune response (Miller et al. 1996). Antigen-independent mechanisms such as different survival of T-cell clones or decreased thymic generation of new naïve T-cells may also influence the clonal composition of peripheral T-cells. These factors may eventually lead to the narrowing of the clonal repertoire and to the appearance of predominant clones in aged people. Both in CD4 and in CD8 T-cells, clonal expansion comprises several TCR V_β families suggesting that a multiplicity of antigenic stimulations are involved in the selection of the expanded clones. The $CD4^+$ T-cell repertoire remains largely polyclonal throughout life, since $CD4^+$ expanded clones accumulate predominantly in the $CD45R0^+$ compartment of exceptionally individuals (centenarians; Wack et al. 1998). On the other hand, $CD8^+$ T-cell subsets contain expanded clones which are already detectable in young adults and become very frequent in older donors both in $CD45RA^+$ and in $CD45R0^+$ compartments. The presence of expanded clones in the $CD45RA^+$ compartment implies that this age-related phenomenon starts earlier, and it is more pronounced in $CD8^+$ than in the $CD4^+$ T-cell subsets indicating that in these two subsets the clonal expansion is controlled by substantially different

mechanisms. Besides, while the finding of expanded CD45R0$^+$ T-cell clones is explained by antigen-driven proliferation, the detection of expanded clones both in CD45RA$^+$ and in CD45R0$^+$ subsets support the idea of reversion from the CD45R0$^+$ to the CD45RA$^+$ phenotype after antigen encounter (Wack et al. 1998). Moreover, TCR V_β repertoire of T lymphocytes was studied in healthy, long-living people and centenarians using a spectra typing method, and expansion of TCR Vβ1, Vβ8, and Vβ20 in long-living people compared with young people was found. In addition these expanded clones were mainly negative for CD28 (Pennesi et al. 1999, 2001) moderate. Indeed, human aging markedly reduces diversity in both CD45RA$^+$ and CD45R0$^+$ CD8$^+$ T lymphocytes thus affecting the cytotoxic compartment in elderly where several compensatory mechanisms may contribute to alleviate the restricted CD8$^+$ T-cell repertoire (increased cross-reactivity of primed CTL clones, increased number of cytolytic CD28$^-$ T-cells or finally increased number of NK cells). Furthermore CD4+ T cell clones derived from centenarians produce mainly Th0 type cytokines with wide effector functions (Wack et al. 1998).

4 Systemic Inflamm-Aging

The inflammatory scenario that characterizes inflamm-aging constitutes a highly complex response to various subtle internal and environmental inflammatory stimuli mediated mainly by the increased circulating levels of pro-inflammatory cytokines. This condition is able to continuously generate Reactive Oxygen Species (ROS) causing both oxidative damage and eliciting an amplification of the cytokines' release, thus perpetuating a vicious cycle resulting in a chronic systemic proinflammatory state where tissue injury and healing mechanisms proceed simultaneously and damages accumulate slowly and asymptomatically over decades. Accordingly inflamm-aging is at the same time a major determinant both of the aging process and of the development of age-associated diseases (Candore et al. 2006; De Martinis et al. 2005; Franceschi et al. 1995; Giunta, 2006; Lio et al. 2003; Vasto et al. 2007). Moreover, the shift of cytokine production toward a pro-inflammatory profile is accompanied by endocrine and metabolic alterations (Paolisso et al. 2000) that could explain some age-related processes such as sarcopenia, obesity, metabolic syndrome and diabetes, among others.

Sarcopenia, i.e. the age-associated decline in skeletal muscle mass, strength and power resulting in physical disfunctioning, contributes to physical inactivity, functional disability and mortality. The specific mechanisms underlying age-related muscle wasting are still largely unknown, although a decreased anabolic state in combination with an increased catabolic state results in a progressive loss of lean tissue. In recent years, the role of inflammatory cytokines in the progression of muscle wasting has been focused (Roth et al. 2006). Recent data support the association between elevated IL-6 levels with in advancing age increased physical decline and mortality. For example, muscle performance measures are significantly lower in hospitalized geriatric patients with high levels of CRP and IL-6 compared with

matched patients with normal levels of inflammation (Bautmans et al. 2005). We evaluated the joint effect of IGF-I and IL-6 on muscle function in a population-based sample of 526 persons with a wide age range (20–102 years). After adjusting for potential confounding factors (age, sex, body mass index), IL-6 receptor, IL-6 promoter polymorphism, IL-6, IGF-I, and their interaction were significant predictors of muscle power. In analyses stratified by IL-6 tertiles, IGF-I was an independent predictor of muscle function only in subjects in the lowest IL-6 tertile, suggesting that the effect of IGF-I on muscle function depends on IL-6 levels. This mechanism may explain why IL-6 is a strong risk factor for disability (Barbieri et al. 2003a). Giresi and colleagues (2005) reported a "molecular signature" of sarcopenia, coming from microarray analyses of young versus old skeletal muscle response. An increased expression of genes involved in the inflammatory was noted within this signature, providing some of the first direct evidence of the role of inflammation in aged muscle changes.

Several papers show data about the importance of TNF-α in muscle wasting. Roubenoff et al. (2003b) reported an association between higher levels of TNF-α and IL-6 with increased mortality in community dwelling elderly, while Yende et al. (2006) observed lower quadriceps strength in older man and woman with high IL-6 and TNF-α levels. Importantly, an interplay between an increase of inflammatory signals and a reduction of opposite growth factors signals may have the most relevance for the progression of muscle wasting. For example, elevated levels of TNF-α and IL-6 have been associated with an increased risk of sarcopenia, frailty and mortality, whereas elevated IGF-I levels have generated opposite associations (Leng et al. 2004; Payette et al. 2003; Roubenoff et al. 2003b).

Recent data on animals and humans indicate a possible more complex role of IL-6. It has been suggested that muscle-derived IL-6 contributes to mediate the beneficial metabolic effects of exercise and may contribute to inhibit TNF-production and thereby insulin resistance (Pedersen and Bruunsgaard 2003). Indeed experimental data indicate that IL-6 is released from skeletal muscle during acute exercise, and its production can result in an increase of antiinflammatory cytokines such as IL-1ra and IL-10 and in a concomitant inhibition of TNF-α (Petersen and Pedersen 2006).

Several studies have investigated the potential relationship between muscle mass and body fatness. How these two components of body composition change with aging, and their combined effects on functional performance and development of frailty, has led to the concept of "sarcopenic obesity" (Baumgartner et al. 2004; Dominguez and Barbagallo 2007; Roubenoff et al. 2004; Zoico et al. 2004). Weight changes are associated with the loss both of fat and lean mass, with the greatest proportion being fat. Individuals with an obesity state associated with high levels of body fat and low levels of muscle mass have an increased risk of functional decline (Baumgartner et al. 2004; Newman et al. 2003; Visser et al. 2002; Zoico et al. 2004) and mortality.

Obesity itself is associated with an elevation of inflammatory markers, and adipose tissue evolved from being identified as a mere deposit of fat as highly metabolically active organ with a critical role in the inflammatory process. In fact, the

current view of adipose tissue is that of a dynamic secretory organ, sending out and responding to signals that modulate appetite, energy eaxpenditure, insulin sensivity, endocrine and reproductive systems, bone metabolism, inflammation and immunity. Mature adipocytes are involved in endocrine, paracrine and autocrine regulatory processes trough the secretion of a large number of multifunctional molecules collectively termed as "adipokines" (Yudkin et al. 1999). In addition to playing roles in the regulation of lipid and glucose homeostasis, adipokines modify some physiological processes, such as hematopoiesis reproduction, feeding behavior and may mediate the genesis of the multiple pathologies associated with increased fat mass (Chaldakov et al. 2003; Rajala et al. 2003). In humans, the development of adipose tissue has been associated with an increased production of inflammatory markers, including adhesion molecules (P-selectin, intercellular adhesion molecule-1, and plasma E-selectin) and inflammatory cytokines (TNF-α, IL-6, IL-8 and MCP-1; Loffreda et al. 1998; Takahashi et al. 2003). It has also been shown that macrophages residing in the adipose tissue may also be a source of proinflammatory factors, such as IL-6 and TNF-α, and that they also may modulate the secretory activity of adipocytes (Xu et al. 2003). It is therefore tempting to speculate that adipocytes, via the production of adipokines, are directly involved in the genesis of systemic and vascular inflammation.

The effects of adipocytokines on vascular function, immune regulation and adipocyte metabolism make them key players in the pathogenesis of metabolic syndrome. Obesity and inflammation have also been associated with the presence of the metabolic syndrome (Aronson et al. 2004; Florez et al. 2006), a cluster of clinical symptoms associated with increased risk of developing cardiovascular disease, diabetes, mortality, and other important adverse health outcomes. The prevalence of metabolic syndrome increases dramatically with age and comprises five cardiovascular risk factors including abdominal obesity, hypertriglyceridemia, low high-density lipoprotein (HDL) levels, hypertension, and hyperglycemia. Insulin resistance is at the basis of most of the features of this syndrome. Given the role of insulin in suppressing several proinflammatory transcription factors, such as NF-kB, Egr-1 and AP-1 (Aljada et al. 2002), an impairment of the action of insulin would result in the activation of these proinflammatory transcription factors, explaining why an insulin-resistant state may be considered proinflammatory (Dandona et al. 2005). High levels of inflammation increase the risk of developing diabetes and atherosclerosis and are thought to be a possible mechanism for the adverse consequence of metabolic syndrome (Barzilay et al. 2001; Pradhan et al. 2001). Whether inflammation leads to metabolic syndrome or vice versa is unclear. Most likely, inflammation and metabolic syndrome are related in a circular process (inflammation leads to metabolic syndrome, and metabolic syndrome increases inflammation; Dandona et al. 2005). In addition, markers of inflammation and several individual components of the metabolic syndrome have been associated with an increased risk of developing dementia and cognitive decline (McGeer EG and McGeer PL 1999, 2004; Yaffe et al. 2003). Most likely, the metabolic syndrome contributes to accelerate atherosclerosis associated with inflammatory response and, in turn, either atherosclerosis or inflammation or both contribute to the

cognitive decline (Yaffe 2007; Grundy 2003; Ridker and Morrow 2003). Insulin resistance and/or hyperinsulinemia associated with metabolic syndrome, increasing systemic inflammatory responses and oxidative stress (Caballero 2004; Parrott and Greenwood 2007), play a central role in increasing central nervous system (CNS) inflammatory markers (Fishel et al. 2005). We showed that independently of age, sex, body mass index, waist-to-hip ratio, triglycerides, drug intake, diastolic blood pressure, smoking habit, and carotid atherosclerotic plaques, higher IL-6 serum concentrations were associated with higher insulin resistance, whereas sIL-6R levels were associated with lower insulin resistance. Furthermore, IL-1ra concentrations were associated with insulin-resistance syndrome, and higher sIL-6R plasma levels continued to correlate negatively with insulin-resistance syndrome (Abbatecola et al. 2004).

Interestingly, increased CNS inflammation has been positively correlated with amyloid-beta (Aβ) levels and insulin-resistant individuals with the highest inflammation exhibit more serious cognitive deficits (Yaffe et al. 2004). This synchronous hyperinsulinemia-induced increase of Aβ and inflammation may represent an important pathway through which insulin resistance promotes both cognitive deterioration and Alzheimer's disease pathology (AD; Craft 2007). Thus, inflammation has been demonstrated to play a role in AD pathogenesis and IL-1 and IL-6 are two of the most important cytokines involved in AD neuro-inflammation (Akiyama et al. 2000; Franceschi et al. 2001; Griffin et al. 2000). In this context, it is important to remember that the biological role of these cytokines in the brain is quite complex, and that their release may directly affect neuronal survival and injury response. In fact, IL-1 and IL-6 may have either trophic or toxic effects. In particular, IL-1 can induce the over-expression of S100β, a neurite growth-promoting cytokine markedly elevated in the brain of AD patients, by reactive astrocytosis. IL-1 can stimulate excessive synthesis, translation and processing of Aβ and plaque associated proteins, and it was shown to lead to over-expression and increased phosphorylation of TAU, thus contributing to an acceleration of degenerative cascades. This cytokine can activate astrocytes and their production of neurotoxic molecules, being astrogliosis a hallmark of AD in the cortex and hippocampus. Concerning IL-6, it appears that microglia, astroglia, neurons and endothelial cells are capable injury response this cytokine, which in turn can induce acute phase proteins. Elevated levels of IL-6 cause significant CNS damage and behavioral deficits (Akiyama et al. 2000). In AD patients, the expression of IL-6 mRNA is increased in brain areas where amyloid deposition and astroglia activation are more prominent (Strauss et al. 1992) and increased IL-6 levels in the brain have been implicated in plaque formation (Huell et al. 1995). Two different polymorphic regions of the IL-6 gene were investigated in patients with AD and nondemented controls (Licastro et al. 2003). The -174 C allele in the promoter region of IL-6 gene was over-represented in AD patients compared to controls, significantly increasing the risk of AD. Moreover, the -174 CC genotype was associated with a high risk of the disease in women. The D allele of a variable number of tandem repeat (VNTR) was in strong linkage disequilibrium with the -174 C allele and slightly increased AD risk. On the other hand, the frequency

of the VNTR C allele decreased in patients with AD and was negatively associated with the risk of developing AD. Both the -174 CC and VNTR DD genotypes were also associated with increased IL-6 levels in blood and brain from AD patients. These findings suggest that IL-6 may play a multifaceted role in AD affecting the turnover of the cytokine.

However, at present, the sources of inflammatory stimuli underpinning and sustaining inflamm-aging are not completely cleared. In addition to the age-related increase of inflammatory compounds occurring the brain (Licastro et al. 2003), adipose tissue, and muscle, it is becoming more and more evident the possible and until now unexplored contribution of other or

Molecular and Cellular Aspects of Macrophage Aging

Carlos Sebastián, Jorge Lloberas and Antonio Celada

Contents

Abbreviations

AML1/CFB	acute myeloid leukaemia/core-binding factor
Atm	ataxia telangiectasia mutated
BMP	bone morphogenetic protein
C/EBP	CCAAT/enhancer-binding protein
CBP	CREB-binding factor
CCR	CC chemokine receptor
dsRNA	double-stranded RNA
EGF	epithelial growth factor
FcγRI	Fc-γ receptor I
GEMM-CFU	granulocyte-erythrocyte-megakariocyte-macrophage colony-forming unit
GM-CFU	granulocyte-macrophage colony-forming unit
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSC	hematopoietic stem cell
IFN-γ	interferon gamma
IκB	inhibitor of NF-κB
IL	interleukin
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCAF	macrophage chemotactic and activating factor
M-CFU	macrophage colony-forming unit

A. Celada (✉) · C. Sebastián · J. Lloberas
Institute for Research in Biomedicine- University of Barcelona
Josep Samitier 1-5, E-08028 Barcelona, Spain
Tel.: +34-93-403 71 65
Fax: +34-93-403 47 47
E-mail: acelada@ub.edu

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M-CSF	macrophage colony-stimulating factor
MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory protein-1
MR	mannose receptor
NER	nucleotide excision repair
NF-κB	nuclear factor-kappa B
NHEJ	non-homologous end-joining
NOS2	inducible nitric oxide synthase
OPG	osteoprotegerin
PASG	proliferation-associated SNF2-like gene
PBMC	peripheral blood mononucleated cell
PKC	protein kinase C
RANKL	soluble receptor activator of NF-κB ligand
ROS	reactive oxygen species
Sir2	silent information regulator 2
SIRT	sirtuin
TARDC	thymus and activation regulated chemokine
TGF	tumor growth factor
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TRAF	TNF-receptor-associated factor
VEGF	vascular endothelial growth factor

Abstract: Macrophages are key cells in innate and adaptive immune function. These cells are involved in the destruction of bacteria, parasites, viruses and tumor cells and lead to the initiation of the inflammatory process. In addition, macrophages are responsible for processing antigens and presenting digested peptides to T-lymphocytes initiating the adaptive immune response. Finally, macrophages participate in the resolution of the inflammatory process by promoting tissue repair. Macrophage functions are affected by aging, thereby contributing to the immunosenescence of adaptive and innate immunity. Here, we summarize data about the effects of aging on macrophages and we discuss the molecular events that could be involved in this process.

Keywords: Aging • DNA damage • Immunosenescence • Inflammation • Macrophages

1 Introduction

Aging can be defined as the time-related deterioration of the physiological functions required for survival and fertility. Among these, immune function has been shown to be dysregulated with advancing age, thus leading to increased susceptibility to viral and bacterial infections, reactivation of latent viruses and decreased response to vaccines (Miller, 1996; Effros, 2001). This impairment of the immune system, called immunosenescence, is associated with increased mortality and major incidence of immune diseases and cancer in the elderly.

Innate and adaptive immunity are compromised by aging. T-cell-dependent and T-cell-mediated functions, such as proliferation, cytotoxicity, cytokine secretion and capacity to respond to novel antigens, are impaired in old age (Fabris et al. 1997; George and Ritter, 1996; Miller, 1996; Pawelec and Solana, 1997). Alterations in B-cells during aging have also been reported. In mice, a progressive decline in germinal centre formation is observed with age (Zheng et al. 1997); the number of circulating $CD27^+$ memory B-cells is reduced in the elderly (Breitbart et al. 2002; Colonna-Romano et al. 2003), and CD40 expression in B-cells is also impaired. Similar to the decline of the adaptive immune system, the functions of NK cells, macrophages and neutrophils also decrease with age (Butcher et al. 2001; Garg et al. 1996; Lloberas and Celada, 2002; Solana and Mariani, 2000), which may explain the increased incidence of bacterial and viral gastrointestinal and skin infections.

Macrophages are key cells in innate and adaptive immune function. These cells may act directly, by destroying bacteria, parasites, viruses and tumor cells, or indirectly, by releasing mediators such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF- α), etc, which can regulate other cells. Macrophages are also responsible for processing antigens and presenting digested peptides to T-lymphocytes, as well as for tissue damage repair. Macrophage functions are altered in old age in humans, mice and rats, thereby contributing to the immunosenescence of adaptive and innate immunity (Lloberas and Celada, 2002). Phagocytic activity, cytokine and chemokine secretion, antibacterial defenses such as the production of reactive oxygen and nitrogen intermediates, infiltration and wound repair function in the late phase of inflammatory response, and antigen presentation, are altered in aged macrophages (Donnini et al. 2002; Herrero et al. 2002; Plowden et al. 2004), which lead to impairment in the first line of immune defense and a decreased capacity to contribute to the development of specific immune responses by presenting antigens to T-cells and by producing regulatory cytokines. Since macrophage activity is essential for the proper function of the immune system, studies regarding the effects of aging on the biology of these phagocytic cells and the molecular mechanisms involved in this process may contribute to a greater understanding of aging and immunosenescence. In addition, it would be of great interest to distinguish between the indirect (i.e., interactions with other cells) and the direct (i.e., genome modifications) effects of aging on macrophage biology to fully understand the macrophage aging process.

2 Macrophages

Macrophages are phagocytic cells involved in a number of complex functions in disease and health. They are critical to the establishment of the immune response against invading pathogens and to the maintenance of homeostasis, by promoting angiogenesis and tissue remodeling and repair. In addition, these cells are responsible for scavenging cellular debris and apoptotic cells (Mantovani et al. 2002).

Under the effect of growth factors, macrophages proliferate but the presence of microbial agents, cytokines or inflammatory molecules blocks this proliferation and induces functional activities (Xaus et al. 1999). This activation leads to the release of toxic metabolites and to the elimination of microbes by phagocytosis (Schroder et al. 2004).

Macrophages, as all blood cells, originate from hematopoietic stem cells (HSCs) in bone marrow under the presence of some growth factors and cytokines. The combined action of interleukin (IL)-1, IL-3 and/or IL-6 induces stem cell division, giving rise to a new stem cell and a pluripotent myeloid cell, also referred to as granulocyte-erythrocyte-megakariocyte-macrophage colony-forming unit (GEMM-CFU). In the presence of IL-1 and/or IL-3, this precursor is committed to becoming a progenitor of both macrophages and granulocytes known as the granulocyte-macrophage colony-forming unit (GM-CFU), which is also committed to the macrophage colony-forming unit (M-CFU) by action of the macrophage colony stimulating factor (M-CSF), the granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3. The M-CFU differentiates, in the presence of M-CSF, into monoblast, promonocyte, monocyte and, finally, into differentiated macrophages.

The differentiation process is regulated by the combined action of several transcription factors (Valledor et al. 1998); among these, PU.1, C/EBP and AML1/CFBβ play a crucial role in regulating the myeloid-specific expression of the M-CSF and GM-CSF receptors required for differentiation, proliferation and survival of mac-

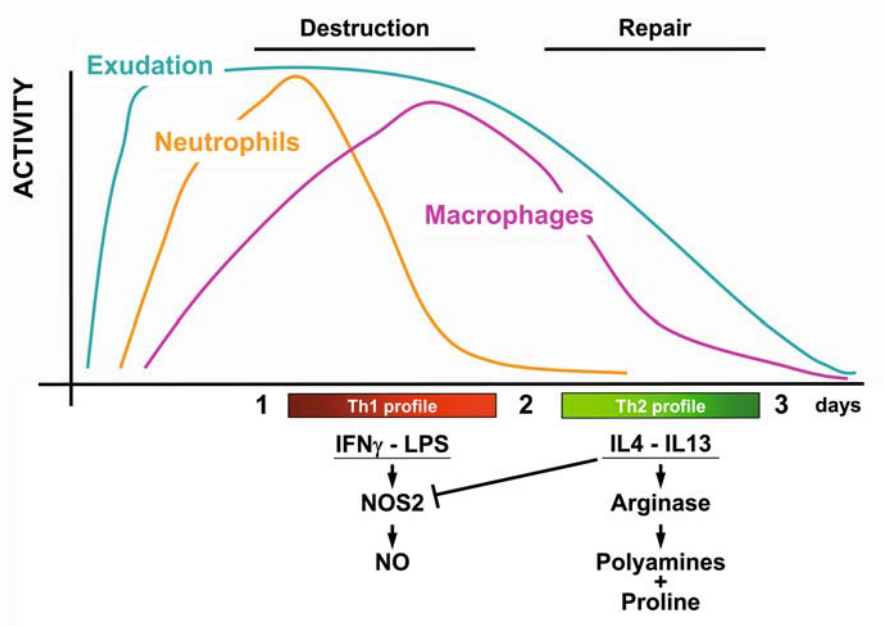

Fig. 1 Macrophages originate from HSC in bone marrow and migrate to body tissues where they become differentiated. Once in the tissues, macrophages may proliferate or become activated during an inflammatory process. However, when not activated, most macrophages die by apoptosis

rophages (Hohaus et al. 1995; Lloberas et al. 1999; Smith et al. 1996). Once in the blood, these cells can migrate to body tissues and differentiate, under the influence of cytokines and depending on the tissue type, into cell types with different functional activities such as osteoclasts (bone), Kupffer cells (liver), microglia (brain), etc. However, when not activated, most macrophages die by apoptosis (Fig. 1).

2.1 Macrophage Functions

Macrophages play a key role in both innate and adaptive immunity. They recognize and destroy invading pathogens and apoptotic cells and modulate the immune response by producing cytokines and chemokines. Moreover, macrophages, as antigen presenting cells, are involved in the regulation of the differentiation and activation of T-cells by the antigen presentation process. In addition to these functions, macrophages play a crucial role in the resolution of inflammation and in tissue repair by promoting synthesis of the extracellular matrix, fibroblast proliferation, angiogenesis and elimination of cellular debris (Rosmarin et al. 1995). Lastly, macrophages eliminate modified proteins, oxidized low density lipoproteins, apoptotic cells and other components from the tissues by expressing scavenger receptors.

Macrophages arriving at the inflammatory loci in the early steps kill remaining microorganisms, remove cell debris and apoptotic bodies and, in a second step, these cells reconstitute damaged tissues (Arnold et al. 2007; Fig. 2). Under the effect of

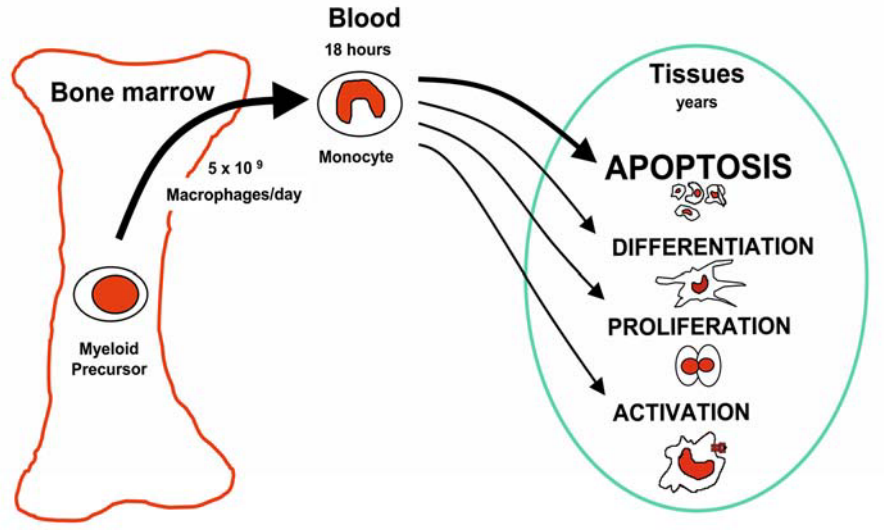

Fig. 2 Macrophages play a key role during the inflammatory process. In the initial phase they are activated in a Th1 context, leading to the release of inflammatory mediators (proinflammatory cytokines and chemokines, nitric oxide, ROS, etc.). In the resolution phase, macrophages become alternatively activated by Th2-type cytokines and participate in tissue repair and remodeling through the production of polyamines and proline

cytokines or bacterial products, macrophages become activated and undergo a series of biochemical, morphological and functional modifications. Th1-type cytokines such as interferon-gamma (IFN-γ) that interacts with its specific receptor, or bacterial products such as lipopolysaccharide (LPS), Gram-positive bacteria and yeast cell wall components, dsRNAs, bacterial flagellin and CpG oligodeoxynucleotides, induce classical activation of macrophages. These molecules are recognized by specific receptors called Toll-like receptors (TLRs; Akira et al. 2001; Alexopoulou et al. 2001; Gewirtz et al. 2001; Hemmi et al. 2002). This activation leads to inflammation and elimination of the pathogen. In addition to this classical activation, also known as M1, it has been reported that several cytokines such as IL-4 and IL-13 induce a distinct alternative activation programme (M2; Gordon, 2003). Recently, it has been shown that IL-21, Activin A and Chitin also mediate alternative macrophage activation (Ogawa et al. 2006; Pesce et al. 2006; Reese et al. 2007). Classical activation is characterized by the expression of inducible nitric oxide synthase (NOS2) and by the biosynthesis and release of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, IL-1 and IL-6. In the alternative activation, the expression of arginase 1 is induced, together with the upregulation of the mannose receptor (MR) and several other markers (Mantovani et al. 2004). Curiously enough, arginine is the substrate for NOS2 and for arginase 1 and the system that transports this amino acid is induced by both types of cytokines providing more arginine inside the cell (Yeramian et al. 2006a, b). NOS2 degrades arginine to produce NO while arginase produces ornithine and polyamines. Alternatively activated macrophages exert immunoregulatory functions, drive type II responses and participate in tissue remodeling.

2.2 Activation of Macrophages

The main activators of macrophages are LPS and IFN-γ. These molecules induce microbicidal and proinflammatory functions in macrophages and, therefore, the destruction of the invading pathogen.

IFN-γ is a type II interferon mainly produced by activated T and NK cells (Imai et al. 1999; Yoshimoto et al. 1998). However, other cell types such as professional antigen presenting cells can also release it (Frucht et al. 2001; Pestka et al. 2004). IFN-γ induces an antiproliferative and antiviral response and is critical to the establishment of the immune response as it promotes the recruitment of lymphocytes at the inflammation site by inducing the production of chemokines and the expression of adhesion molecules (Puddu et al. 1997). Moreover, IFN-γ leads to the expression of several genes that regulate many aspects of macrophage biology. It induces the expression of the Fc high affinity receptors (FcγRI) in the cell surface leading to increased antibody-dependent cytotoxicity (Vaday et al. 2001); it increases the phagocytic activity of macrophages; it induces a respiratory burst (generation of nitric oxide and reactive species of oxygen) and the expression of lysosomal enzymes promoting the destruction of the pathogen (Capsoni et al. 1994). IFN-γ inhibits M-CSF-dependent proliferation and protects macrophages from apoptosis

induced by glucocorticoids or M-CSF withdrawal. This protective effect of IFN-γ is mediated by $p21^{waf1}$ expression and blockade of the cell cycle at the G1/S boundary (Xaus et al. 1999). We have observed that in granulomas, where macrophages need to survive for a longer time, there are increased levels of IFN-γ correlating with increased levels of $p21^{waf-1}$ (Xaus et al. 2003). In addition to modulating the innate immunity, IFN-γ regulates the adaptive immunity by regulating the expression of the major histocompatibility complex (MHC) class II genes at several levels (Cullell-Young et al. 2001; Gonalons et al. 1998), which are crucial for presenting antigens to T-lymphocytes and for initiating an immune response.

The effect of LPS on macrophage function is mediated by the interaction with its receptor, the Toll-like receptor 4 (TLR4). Activation of macrophages by LPS leads to an increase in mRNA synthesis and to the secretion of proinflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-8, IL-12, TGF-β and the macrophage inhibitory factor (MIF). Moreover, in response to LPS macrophages release arachidonic acid metabolites (e.g., platelet-activating factor, prostaglandin and leukotriens), proteases, eicosanoids, nitric oxide and other reactive oxygen species (ROS; Miller et al. 2005; Muzio et al. 1997). All these cytokines and mediators are critical to the initiation of inflammatory response and contribute to the efficient control of growth and dissemination of invading pathogens.

In addition to this classical activation, macrophages can be activated by Th2 cytokines acquiring an M2 phenotype. Alternative activation of macrophages by IL-4 and IL-13 produces M2-type responses, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens. This alternative activation results in the up-regulation of the expression of the MR and MHC class II molecules, which stimulates endocytosis and antigen presentation, respectively. These cytokines also induce the expression of selective chemokines such as macrophage-derived chemokine (MDC, also known as CCL22) and thymus and activation regulated chemokine (TARDC, CCL17), and intracellular enzymes, such as arginase, that are involved in cell recruitment and repair of granuloma formation, thereby counteracting the effects of the inducible NOS2 activation and nitric oxide release (Gordon, 2003). Moreover, the induction of arginase down-regulates the expression of NOS2 at the translational level (Lee et al. 2003). The catabolism of arginine by arginase produce l-ornithine and ultimately polyamines that induce fibroblasts proliferation and collagen production.

3 Aged Macrophages

Macrophages from aged humans and mice display several defects in their function. Many studies have focused on the effects of aging on macrophage biology but have yielded conflicting, and sometimes opposing, results. This may be due to factors such as the strain and sex of experimental subjects, distinct macrophage origin (bone-marrow, peritoneum, spleen, or alveolus) and differences in experimental conditions (culture, stimulant used, etc.). Furthermore, in the case of humans, it is difficult to define the term ``healthy elderly subject,'' which implies careful

screening for health. Furthermore, most studies on humans have been performed with monocytes, which generally provide a limited view of tissue macrophages. In addition, the majority of studies on macrophage aging have shown modifications in their functional activities; however, in few cases these studies have provided an explanation of the basis of this dysfunction.

3.1 Differentiation and Maturation of Macrophages

The immune system is maintained by the generation of immune cells from HSCs. These cells reside in the bone marrow and provide lifelong production of progenitors and peripheral blood cells. Simultaneously, HSCs must be able to maintain the stem cell pool by selfrenewal divisions. Increasing experimental evidence supports the premise that HSCs become aged and have a limited functional lifespan (Geiger and Van Zant, 2002). The first studies to suggest stem cell aging involved serial transplantation of whole bone marrow that supported only 4 to 5 rounds of transplantation (Harrison and Astle, 1982; Van Zant and Liang, 2003). Given that the HSC compartment facilitates this regeneration, these findings suggested an exhaustion of the stem cell pool. In fact, there is ample evidence that stem cell quality decreases with each selfrenewal division (Van Zant et al. 1997). Mouse experiments revealed that the number of HSCs increased while their proliferative capacity decreased with age (de Haan and Van Zant, 1999; Morrison et al. 1996). Results from studies comparing HSCs in different mouse strains indicate that HSC functional decline can be correlated with lifespan. In addition, a negative correlation has also been shown between lifespan and proliferative capacity (de Haan et al. 1997; Geiger and Van Zant, 2002). Progenitor cells from long-lived C57BL/6 mice have a relatively low cycling activity, whereas the stem cell pool increases with age and is relatively small. In contrast, DBA/2 mice have a shorter lifespan than C57BL/6 mice, their progenitors show increased cycling activity, and their stem cell pool decreases upon aging and is relatively large (de Haan et al. 1997). All this suggests that rapidly dividing cells exhaust faster.

But, how does the aging of HSCs affect the generation of macrophages? To date, it is not clear whether the generation of macrophages from their precursors is impaired with aging. In humans, there is a reduction of CD68-positive cells, which are markers of macrophage population (Ogawa et al. 2000). The percentage of CD68-positive cells is high in children (first and second decades) and then decreases as the individual gets older. Moreover, it has been hypothesized that this reduction in the macrophage population may have an influence on the reduction of HSC proliferation and on the induction of apoptosis in the bone marrow of elderly people, probably via reduced production of growth factors and cytokines (Arkins et al. 1993; Kelley et al. 1996; Minshall et al. 1997). By contrast, macrophages increase in density in myeloproliferative disorders suggesting that there was a correlation between macrophage density and myelopoietic activity (Sadahira et al. 1999). In mice there are conflicting data. According to Wang et al. (1995), the

macrophage population is enhanced in bone marrow, as shown by an increase in Mac1-positive cells. This is reflected as an increase with age in the macrophage colony forming unit (M-CFU). Moreover, macrophages from bone marrow of old mice generate less TNF-α than macrophages from young mice suggesting that the increase in the number of macrophages may reflect a compensation for their reduced function. However, we have found that the number, size, DNA content and cell surface markers expressed during macrophage maturation, such as Mac1, were similar in macrophages from aged and young mice (Herrero et al. 2001). Recently, Rossi et al. (2007) have demonstrate that accumulation of DNA damaged has a profound impact on the functional capacity of HSCs with age, leading to loss of reconstitution and proliferative potential, diminished selfrenewal, increased apoptosis and, ultimately, functional exhaustion. In transplantation experiments, it has been shown that recipients transplanted with HSCs from mice deficient in several genomic maintenance pathways have a marked decrease in reconstitution of B-cells, T-cells and myeloid cells. Moreover, these authors provide evidence that endogenous DNA damage accumulates with age in wild-type stem cells. This suggests that an impaired functional capacity of HSCs accumulating DNA damage may derive in a deficient generation of blood cells.

3.2 Effects of Aging on Macrophage Functions

A great number of macrophage functions including phagocytosis, antibacterial defenses, chemotaxis, wound repair and activation have been reported to be altered in human, rats and mice during aging, thereby contributing to the immunosenescence of adaptive and innate immunity (Table 1).

Table 1 Effect of aging on macrophage function

Function	Change with aging
IFN-g activation	
Production of ROS	Decreased
Production of NO	Decreased
Activation of MAPK	Decreased
Expression of MHC II	Decreased
Production of PGE2	Increased
LPS activation	
Production of proinflammatory citokines	Decreased
Production of chemokines	Decreased
Production of ROS	Decreased
Production of NO	Decreased
Expression of TLR4	Decreased or no change
Activation of MAPK	Decreased
Phagocytosis	Decreased
Wound repair	Decreased
Chemotaxis	Decreased

3.2.1 Phagocytosis

Phatocytosis constitutes the first step of immune defense against invading pathogens. Tissue macrophages, alveolar macrophages and polymorphonuclear leucocytes in the blood have all phagocytic activity. However, the data available addressing the effect of aging on the phagocytic function of macrophages and monocytes is unclear. An age-related decline in phagocytosis by neutrophils but not by alveolar macrophages was observed in rats (Mancuso et al. 2001). However, several reports using murine models indicate a decline in the adherence, opsonization, tumor cell killing and phagocytosis by peritoneal macrophages (De La Fuente, 1985; De la Fuente et al. 2000; Khare et al. 1996). In addition, it is observed that the phagocytic activity of macrophages from aged individuals declines in parallel with reduced production of macrophage-derived chemokines (Swift et al. 2001). Altered expression and function of receptors involved in the phagocytic process and their signal transduction may explain the observed reduced phagocytic ability in aging models. However, the effect of aging on these proteins has not been reported.

3.2.2 Chemotaxis

To eliminate invading pathogens macrophages must migrate toward the inflammation site in a process controlled by chemotactic stimuli. The main chemotactic factors are chemokines secreted by the endothelium, neutrophils, T-cells, monocytes and macrophages, such as macrophage chemotactic and activating factor (MCAF), macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES and IL-8 as well as complement products such as C5a, C3a and C4a. A reduction in the production of MIP-1α and MIP-1β by macrophages from aged mice has been described (Ashcroft et al. 1998). Moreover, the chemotactic response of macrophages to complement-derived factors is impaired in elderly individuals (Fietta et al. 1993). Aschroft et al. (1998) collected coetaneous punch biopsies of the wounds from 138 healthy subjects, aged 19–96 years at fixed time-points from day 1 up to 3 months postwounding. Using quantitative imaging, they demonstrated that monocyte/macrophage and lymphocyte appearance was delayed in the aged individuals. Thus, these data suggest that aged macrophages show an impaired chemotactic response that may contribute to delayed pathogen clearance in healthy elderly individuals.

3.2.3 Activation of Macrophages

The different aspects of macrophage classical activation is the most studied effect of aging on macrophages. IFN-γ activation is impaired in aged macrophages. Studies using rats have demonstrated a 75% decrease in the capacity of macrophages from aged animals to produce superoxide anion after incubation with IFN-γ or opsonized zymosan (Davila et al. 1990). Furthermore, the production of peroxide and nitric oxide in response to IFN-γ by peritoneal macrophages is diminished in aged mice (Ding et al. 1994). This was explained by a reduced IFN-γ induced mitogen-activated protein kinase (MAPK) phosphorylation in macrophages from aged mice.

In addition to microbicidal activities, IFN-γ induces the expression of MHC class II molecules that are involved in the initiation of the adaptive immune response. Antigen presentation by macrophages is decreased with age, possibly due to diminished expression of MHC class II molecules both in human and mice (Herrero et al. 2001; Plowden et al. 2004). We have found that bone marrow macrophages from aged mice express half of the MHC class II antigen IA molecules at the cell surface when stimulated with IFN-γ (Herrero et al. 2001). IAβ mRNA expression is also lower in aged macrophages because there is a smaller amount of transcription factors that bind to the W and X boxes of MHC class II gene promoter. In addition, it has been shown that human monocytes express decreased levels of HLA-DR/DP (Villanueva et al. 1990). Moreover, activated macrophages from aged humans and mice produce higher amounts of prostaglandin E2 than younger individuals, which inhibits surface expression of MHC class II, thus contributing to the decreased capacity of antigen presentation of macrophages observed with age (Plowden et al. 2004).

Activation by LPS is also altered in aged macrophages. Although inflammatory cytokines are elevated in the plasma of aged animals and humans (Franceschi et al. 2000; Saurwein-Teissl et al. 2000), the production of inflammatory cytokines by peritoneal macrophages from rats and mice decreases with age. Stimulation of macrophages from aged rodents with LPS results in significantly lower production of IL-1, TNF-α, and IL-6 (Inamizu et al. 1985; Plackett et al. 2004; Wallace et al. 1995), as well as lower production of chemokines, such as MIP-1α and MIP-1β (Swift et al. 2001). The production of oxidative radicals in response to LPS also appears to decline with age, and the expression of NOS2 and the production of nitric oxide are reduced in macrophages from aged rodents (Alvarez et al. 1996; Khare et al. 1996; Kissin et al. 1997; Plackett et al. 2004).

There is some controversy concerning the basis for the decline in the production of inflammatory cytokines and oxidative radicals in response to LPS stimulation. Renshaw et al. (2002) found that expression of a variety of TLRs, including TLR4, was decreased in the aged, which could be the reason for a decreased response of macrophages from aged mice to LPS. Conversely, Boehmer et al. (2004) did not find a reduction in TLR expression and they attributed the impaired cytokine production to a decrease in c-jun N-terminal kinase (JNK) and p38 MAPK activation in macrophages from aged mice. In humans, the decreased response of monocytes to LPS has been associated with deficiencies in the activation of protein kinase C (PKC)-α, PKC-βI and PKC-βII, MAPK and deficient expression of c-Fos and c-Jun (Delpedro et al. 1998). Using a microarray analysis on RNA from resting and LPS-stimulated macrophages from aged and control mice, Chelvarajan et al. (2006) demonstrated that immune response (proinflammatory chemokines, cytokines and their receptors) and signal transduction genes (TLR and MAPK pathways) were specifically reduced in aged mouse macrophages. In addition to reduced levels of IL-1β, IL-6, IL-12 and TNF-α, they found a decrease in IFN-γ, M-CSF, GM-CSF and bone morphogenetic protein-1 (BMP-1) production in aged macrophages. Moreover, many chemokines involved in innate immunity and inflammation are reduced in macrophages from aged mice, such as CCL4, CXCL1, CCL6, CCL9 and CCL24, as well as the receptors CC chemokine receptor 3 (CCR3) and CCR5, involved in chemotaxis of neutrophils, macrophages and eosinophils (Chelvarajan et al. 2006). All this

correlates with the reduction in the overall inflammatory response in spleens from aged mice. Furthermore, a variety of chemokines and receptors (CXCL9, CXCL10, CXCL11, CCR7), which affect CD4 and CD8 T-cell migration and T helper cell type 1 (Th1) development, are reduced in macrophages from aged mice (Chelvarajan et al. 2006). This is in agreement with an age-associated decrease in T-cell function and in particular, Th1 cell function. Several components of the TLR pathway [TNF-receptor-associated factor 6 (TRAF6), CD14, Rel, RelB and some of the subunits of the NF-κB transcription factor] have reduced levels in LPS-stimulated aged macrophages. As this pathway is known to be critical for the production of chemokines and proinflammatory cytokines, these authors conclude that reduced levels of the components of TLR pathway could explain the impaired production of several cytokines and chemokines in LPS-stimulated macrophages from aged mice. In addition to TLR pathway, they also found an increase in the expression and phosphorylation of p38 MAPK in aged macrophages. Low doses of a p38 MAPK inhibitor enhanced proinflammatory cytokine production by macrophages indicating that p38 MAPK activity has a role in cytokine dysregulation in aged mouse macrophages. This is in contrast with the results of Boehmer et al. (2004; *See* above). This discrepancy could be the result of the use of thioglycollate-induced peritoneal macrophages in the Boehmer study versus macrophages from spleen in the Chelvarajan study.

There are few data regarding the way in which aging may affect the alternative activation of macrophages. However, alterations in cytokine secretion by T-cells could affect this process. In mice infected with *S. mansoni*, the production of Th2 cytokines is lower in aged BALB/c animals compared to young ones (Smith et al. 2001). Moreover, older IL-4-/- BALB/c mice express a transient resistance to *L. major* infection, indicating that these animals have a lower capacity for Th2 response (Kropf et al. 2003). Arginase expression, which may play a crucial role in M1/M2 polarization, is also affected by age. Total arginase activity in the postrhinal cortex and in some regions of the hippocampus decreases in aged mice (Liu et al. 2003a, b). However, it has been shown that insulin augments alternative activation of macrophages by IL-4 (Hartman et al. 2004; Liang et al. 2004). As insulin blood levels and insulin resistance increase with age (Petersen et al. 2003), it is tempting to speculate that alternative activation of macrophages may increase during aging. Moreover, the insulin pathway regulates the lifespan in worms, flies and mammals (Tatar et al. 2003). Mutations in some of the components of this pathway leads to an extension of the lifespan of these species (Kenyon, 2005) suggesting that increased insulin signaling may be related to aging. However, further studies are required to examine whether changes in macrophage polarization with aging are responsible for some aspects of immunosenescence.

3.2.4 Wound Repair

In addition to their crucial role in the initial phases of the inflammatory response, macrophages develop important functions in the removal and regeneration of the damaged tissue by secreting angiogenic and fibrogenic growth factors. Studies in human and rodent species have shown an age-related decline in the coetane-

ous wound repair process, which impacts on the inflammatory response and the growth phase of the repair process (Gosain and DiPietro, 2004; Thomas, 2001). These changes include enhanced platelet aggregation, delayed re-epithelialization, delayed agiogenesis, delayed collagen deposition, turnover and remodeling, delayed healing strength, decreased wound strength, and delayed infiltration and function of macrophages. Using a murine model of excision wound repair, Danon et al. (1989) demonstrated that repair and re-epithelialization processes were delayed significantly in aged mice and that the rate of wound repair could be partially restored by the addition of peritoneal macrophages from young mice. In addition, the rates of collagen synthesis and angiogenesis [attributed to a decrease in the secretion of vascular endothelial growth factor (VEGF)] were delayed. TLRs 2, 4, 7, and 9 and adenosine A (2A) receptors mediates the production of VEGF and other angiogenic factors by macrophages (Olah and Caldwell, 2003; Pinhal-Enfield et al. 2003). Hence, the observed decrease in TLR function in aging may contribute to delayed wound healing. Furthermore, the expression of cell adhesion molecules on the vascular endothelium is decreased in the elderly (Ashcroft et al. 1998), and responsiveness (receptor expression) to VEGF and epithelial growth factor (EGF) is reduced (Ashcroft et al. 1997; Kraatz et al. 1999). Thus, the communication between tissue cells and the innate immune system appears impaired, contributing to the observed functional deficiencies in tissue repair.

3.3 Effect of Aging on Tissue-Specific Macrophages

In addition to studies regarding the effect of aging on macrophage biology, many reports have focused on the impact of aging on some tissue-specific macrophages. Thus, alteration in the function of these macrophages may contribute to the pathologies observed in these tissues during the aging process.

Macrophages are dispersed throughout the body. Some take up residence in particular tissues becoming fixed macrophages which serve different functions in different tissues and are named to reflect their tissue location: alveolar macrophages in the lung, thymic macrophages in the thymus, histiocytes in connective tissues, Kupffer cells in the liver, mesangial cells in the kidney, osteoclasts in bones, Langerhans' cells (LCs) in the skin and microglia in the brain.

LCs were originally described as an epidermal macrophage population containing large granules and capable of phagocytosis (Hume et al. 1983; Ralfkiaer et al. 1985). Later, LCs were typed as immature dendritic cells since they can migrate after activation from the skin to regional lymph nodes, a hallmark characteristic of dendritic cells (Cumberbatch and Kimber, 1992; Yamazaki et al. 1998; Wang et al. 1999). Although both macrophages and LCs belong to myeloid lineage, the precise lineage relationship between them is not yet clear. The number of epidermal LCs and their function is diminished as a result of the aging process in humans and mice (Bhushan et al. 2002; Thiers et al. 1984). However, it is not clear whether this defect is a consequence of diminished bone marrow precursor production. These

age-related changes may contribute to altered coetaneous immune function, such as poor or variable contact hypersensitivity to allergens in the elderly.

The functional capacity of Kupffer cells is also impaired in aged mice. They have a substantial reduction in their respiratory burst activity, lessened endocytic capacity and enhanced oxidative stress (Videla et al. 2001).

Among the most striking changes that occur with age is thymic involution, which correlates with the observed impairment of T-cell immunity. This decrease in thymus size is also associated with alterations in thymus architecture (Aspinall, 1997; Bertho et al. 1997). However, little information is available on macrophages during age-dependent thymus involution. In mice, relatively early in the involution process, the number of macrophages and their phagocytic activity increases, with these cells appearing to have a large number of phagolysosomes containing cellular material at various stages of lysis (Hirokawa, 1977; Nabarra and Andrianarison, 1996). This correlates with the decrease in thymocyte numbers (Aspinall, 1997). Over time the number of thymic macrophages diminishes gradually (Nabarra and Andrianarison, 1996; San Jose et al. 2001), in correlation with a reduction in the total number of macrophage precursors and their capacity to proliferate (Zeira and Gallily, 1990). In addition, Varas et al. (2003) demonstrated that the thymic macrophages phenotype (expression of cell surface markers and chemokine receptors) is unaltered in the elderly suggesting that their functional properties on T-cell stimulation, adhesion and migration would also be unimpaired.

Microglia cells are the small, highly ramified immune sentinels of the brain. These cells are distributed throughout the brain parenchyma and are continuously sensing the microenvironment in search for injuries or pathogens (Davalos et al. 2005; Nimmerjahn et al. 2005). After activation, microglia initiate an innate immune response by producing proinflammatory cytokines. Different lines of evidence from humans and mice suggest that senescence of microglia does occur leading to neurodegeneration. Proliferation of microglia during activation is not impaired by old age. In fact, microglia appear to proliferate even more vigorously in older rats after a facial nerve lesion (Conde and Streit, 2006). Moreover, human and rodent microglia show signs of aging-related structural and morphological deterioration (Streit, 2006). The incidence of dystrophic microglia increases in older individuals, supporting the idea that dystrophy is a reflection of cell aging. It is suggested that the deterioration of microglia may be involved in the pathogenesis of neurodegenerative disease, perhaps through progressive loss of microglial neuroprotective capacity (Streit, 2002). In addition to this structural alteration, microglia from healthy aging brains show an increased expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12; Sierra et al. 2007). The higher levels of these cytokines produce tissue degeneration (Aloisi, 2005), and thus the increased levels in aging microglia could contribute to brain damage during aging, and even contribute to the onset of neurodegenerative diseases (Mrak and Griffin, 2005).

There are few data regarding how aging affects the function of osteoclasts. Bone mass is maintained by a delicate balance between formation and resorption. At cell level, the rates of bone formation and resorption reflect the number and activity of stromal/osteoblastic cells and osteoclasts, cells of macrophage origin. Stromal/oste-

oblastic cells regulate the number and activity of osteoclasts through expression of the soluble receptor activator of NF-κB ligand (RANKL), M-CSF and osteoprotegerin (OPG; Cao et al. 2005). With advancing age, expression of RANKL in whole bone and in culture marrow cells from both, humans and animals, gradually increases, and expression of OPG either decreases or remains unchanged. RANKL expression is also increased in early stromal/osteoblastic cells from aged mice (Cao et al. 2003; Fazzalari et al. 2001; Ikeda et al. 2001; Makhluf et al. 2000). Furthermore, the osteoclast progenitor pool is reported to increase with advancing age in mice (Perkins et al. 1994). Cao et al. (2005) showed that aging significantly increases stromal/osteoblastic cell-induced osteoclastogenesis, promotes expansion of the osteoclast precursor pool and alters the relationship between osteoblasts and osteoclasts. Coincident with these changes, the efficacy of osteoclasts to form bone is also impaired. All these modifications may contribute to the osteoporosis associated with aging.

In summary, the aging process has an impact on the function of macrophages and tissue-specific macrophages, thus leading not only to an impaired immune response but also to the development of several pathologies in the tissues where they reside.

4 Molecular Mechanisms Involved in Macrophage Aging

The data presented so far indicates an age-associated malfunction of macrophages. Most of these publications describe the events but do not shed light on the origin of this malfunction. Many theories have been formulated to explain the aging process. Because immunosenescence is a hallmark of aging, these theories may also explain the changes that occur in the immune system as a result of maturation (Fig. 3).

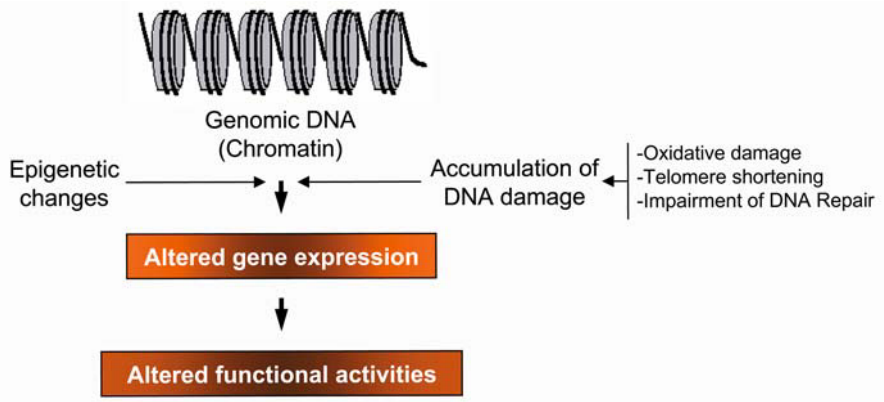

Fig. 3 Molecular view of macrophage aging. Altered gene expression caused by accumulation of DNA damage and by epigenetic changes may, in part, explain the altered functional activities observed in aged macrophages

4.1 Aging and Altered Gene Expression

Aging has been associated with changes in gene expression (Kanungo, 1975). Many genes show an altered expression in several cell types contributing to the observed modification of some functional activities during aging. Loss of the expression of several genes occurs in immune cells. For instance, in T-cells, loss of expression of CD28 (Effros et al. 1994) and IL-2 receptor is related to a deficient co-stimulatory signal and poor proliferative responses. As discussed above, aged macrophages also have altered expression of many genes (TLRs, proinflammatory cytokines, chemokines, MHC class II molecules, signal transduction molecules, transcription factors, etc), which may explain the loss of some functional activities. The molecular basis of the altered expression of some of these genes is related to an impaired signal transduction (MAPK, PKC; Boehmer et al. 2004; Delpedro et al. 1998). In other cases, changes in gene expression result from age-related modifications of one or more transcriptional factors. For example, we have demonstrated that loss of MHC class II expression in aged macrophages was due to lower levels of transcription factors that bind to the promoter of these genes, indicating reduced binding efficiency (Herrero et al. 2001). Moreover, changes in gene expression may be due to epigenetic mechanisms. It has been reported that methylation of CpG islands decreases during cellular senescence and aging (Hornsby et al. 1992; Singhal et al. 1987) and that the activity of the DNA-methyl transferase is also lower in senescent cells (Vertino et al. 1994). In addition, disruption of PASG (lsh), a SNF2-like factor that facilitates DNA methylation, causes premature aging in mice (Sun et al. 2004), which suggests that DNA methylation is essential to maintain the expression patterns required for normal growth and longevity. Furthermore, acetylation and deacetylation of histones are involved in cell senescence (Howard, 1996; Ogryzko et al. 1996; Villeponteau, 1997). In mammals, the histone acetyl transferase activity of p300/CBP is reduced in several tissues in aged mice (Li et al. 2002) and its expression is impaired in neurons of aged rats (Matsumoto, 2002). Moreover, the histone deacetylase Sir2 and its homologs in mammals SIRT1 and SIRT6 are involved in regulation of genomic stability and aging in yeast, worms and mice (Chua et al. 2005; Hekimi and Guarente, 2003; Mostoslavsky et al. 2006). On the basis of these observations, it is of interest to study the epigenetic regulation of gene expression during aging in macrophages.

4.2 Telomere Shortening

Telomeres are chromatin structures that cap and protect the end of chromosomes. In vertebrates, they are formed by tandem repeats of hexamer sequences (TTAGGG) that are associated with various specific proteins (Blackburn, 2001; Chan and Blackburn, 2002; de Lange, 2002) involved in the maintenance and regulation of

telomere length. With selfreplication, telomeres lose TTAGGG repeats because conventional DNA polymerases are not able to completely replicate linear chromosomes (Lansdorp, 2005). Progressive telomere shortening has detrimental implications; chromosome caps are unprotected leading to genomic instability and cell death (Blackburn, 2001; McEachern et al. 2000). However, in normal cells, telomere erosion initiates a cell senescence program which prevents further divisions, thereby protecting cells from excessive telomere loss and cell death (Blackburn, 2001; McEachern et al. 2000).

Telomere shortening has been involved in the aging process and in the regulation of replicative lifespan (Iwama et al. 1998). Late generations of the telomerase KO mice, Terc$^{-/-}$, show severe telomere dysfunction characterized by critically short telomeres and end-to-end fusions. These mice suffer from various age-related diseases that affect highly proliferative tissues (Blasco, 2002). Among these, the generation and function of immune cells has been shown to be affected by telomere attrition. Numerous studies have confirmed that loss of telomeric DNA with progressive telomere shortening occurs in cells of the hematopoietic system as a function of normal replicative aging. Age-dependent loss of telomeric DNA was demonstrated in both neutrophils and lymphocytes (Hastie et al. 1990; Vaziri et al. 1993). Moreover, reduced proliferative capacity of T- and B cells has been described in Terc$^{-/-}$ mice (Blasco, 2002). However, no direct assessment of aged-induced changes in telomere length in monocytes and macrophages has been performed to date. Several studies using peripheral blood mononucleated cells consisting of 10–15% monocytes, 60–70% lymphocytes and 30–15% granulocytes, have shown that these structures shorten with age at a rate comparable to that of purified lymphocytes (Weng, 2001). Mature monocytes do not undergo further cell division after activation. Thus, the variations in telomere length in monocytes as the aging process advances may reflect changes in telomere length in hematopoietic progenitor cells. In fact, HSCs show telomere shortening during in vitro culture and in vivo aging (Engelhardt et al. 1997; Vaziri et al. 1994; Zimmermann et al. 2004). HSCs derived from human and mice lose telomeric DNA with age despite the presence of detectable telomerase activity (Allsopp et al. 2001; Vaziri et al. 1994). Moreover, telomere shortening occurs during serial transplantation of HSCs, coinciding with impaired function (Allsopp et al. 2001). This suggests that telomere attrition may alter the HSC capacity to generate blood cells. In support of this notion, HSCs from telomerase-deficient mice whith short telomeres show a reduced ability to repopulate irradiated mice (Allsopp et al. 2003; Samper et al. 2002).

4.3 DNA Damage

Accumulation of DNA damage may also explain the aging process. Increasing experimental data suggest that somatic mutations accumulate during aging (Curtis and Crowley, 1963; Ramsey et al. 1995; Tucker et al. 1999) and that this accumulation increases exponentially (Martin et al. 1996). This may be due to

an increase in the number of mutations or to a deficient repair activity. DNA damage produced by these mutations may cause an alteration in gene expression patterns, the generation of modified proteins and the alteration of some cellular functions. To repair this DNA damage, cells have developed a DNA damage response which includes the detection of the lesion, the activation of cell cycle checkpoints and the activation of several repair mechanisms to eliminate the damage (Sancar et al. 2004). Deficiencies in some of the components of the DNA damage response leads to senescence and premature aging (Lieber and Karanjawala, 2004) supporting the idea that accumulation of DNA damage is involved in the aging process.

An important mechanism that leads to a wide spectrum of intracellular damage during aging is extended exposure to ROS generated by cellular metabolism (Kregel and Zhang, 2007). It has been long recognized that high levels of ROS can inflict direct damage on macromolecules such as lipids, nucleic acids and proteins impairing their function (Blumberg, 2004). In the hematopoietic system, stem cell functional capacity is severely affected by accumulation of DNA damage (Nijnik et al. 2007; Rossi et al. 2007). Alterations in telomere length maintenance and in the nucleotide excision repair (NER) and non-homologous end-joining (NHEJ) repair pathways limit stem cell function in an age-dependent manner by intrinsically diminishing selfrenewal and proliferative capacity of HSCs. Moreover, elevated levels of ROS are involved in the impairment of HSC function. Studies in mice deficient for the ataxia telangiectasia mutated (*Atm*) gene show that the selfrenewal capacity of HSCs depends on *Atm*-mediated inhibition of oxidative stress. *Atm*-deficient mice show progressive bone marrow failure resulting from a defect in HSC function that is associated with elevated ROS (Ito et al. 2004). Therefore, DNA damage- and ROS-dependent HSC failure may lead to an impaired generation of blood cells and, among these, macrophages during the aging process.

Few reports have assessed the direct influence of DNA damage and ROS on macrophage biology. Activation of macrophages leads to an increase in ROS and nitric oxide production as well as many proinflammatory cytokines that result in the clearance of the invading pathogen. However, this pro-oxidant environment may also cause DNA damage in macrophages themselves, including the induction of apoptosis (Xaus et al. 2000), suggesting that having very efficient antioxidant defenses could be very important for these cells. In this regard, it has been shown that the levels of antioxidant defenses, such as superoxide dismutase activity, decrease with aging in macrophages (de la Fuente et al. 2004), although no data about DNA damage in these cells has been reported.

In addition, elevated levels of ROS modulate some redox-sensitive transcription factors (Kregel and Zhang, 2007). Among these, NF-κB is very relevant because it is a key regulator of macrophage biology. It is thought that the phosphorylation of IκB, the inhibitory subunit of NF-κB, is the key step in NF-κB redox activation. ROS-mediated phosphorylation of IκB, leading to its ubiquitination and degradation, allows the NF-κB complex to be translocated to the nucleus and act as a transcriptional activator (Piette et al. 1997). On the other hand, direct oxidation of critical cysteine residues in the p50 subunit of NF-κB

decreases its DNA binding activity (Piette et al. 1997). It has been reported that macrophages suffer from oxidative stress with aging as reflected by an increase in the oxidized glutathione/reduced glutathione ratio (de la Fuente et al. 2004). Thus, alteration of redox status in macrophages during aging may alter the activity of NF-κB and the expression of its target genes which may lead to the loss of some functional activities.

5 Conclusions and Perspectives

Macrophages are a key component of both innate and adaptive immunity and are of outmost importance in the elimination of an invading pathogen, the initiation of an immune response by activating T-cells and in the resolution of inflammation and tissue repair. Among the physiological functions that are affected by aging, the deterioration of the immune system, called immunosenescence, represents a hallmark of the aging process and contributes to the increased mortality and major incidence of immune diseases and cancer observed in the elderly. Because of the importance of macrophages in the immune system, the altered functions of these cells as a result of the aging process may play a key role in immunosenescence.

Here, we have summarized increasing experimental data about how aging affects macrophage functions. We, and many other authors, have described that most of these functions are altered in aged humans, rats and mice suggesting that dysfunctional macrophages may be involved in the deterioration of the immune system with aging. However, most of these studies have used peritoneal macrophages or blood monocytes which may be influenced by their interaction with other cell types that are also affected by aging, thereby providing a limited view of macrophage aging. On the other hand, the use of bone-marrow derived macrophages represents an extraordinary model to study the effect of aging on the genomic expression of macrophages without the influence of other cell types but does not reflect the precise function of macrophages in vivo in the tissues. Therefore, the integration of data from all macrophage models provides the best strategy to assess how aging affects macrophage function and the molecular mechanisms involved in this process.

Many theories have been postulated to explain the aging process. Even though these theories have been demonstrated in many cell types, very few data are available regarding the cellular and molecular mechanisms involved in macrophage aging. It would be of great interest to study telomere shortening and telomerase activity in aged macrophages as well as the influence of accumulation of ROS and DNA damage in these cells with aging because these studies could probably shed light on the origin of macrophage dysfunction with aging. In summary, a great amount of data demonstrates that macrophage functions are altered by aging contributing to immunosenescence. However, an integrative model which includes all macrophages subsets and a more profound study of the molecular mechanisms involved in this process would be necessary to gain further insight into macrophage aging and immunosenescence.

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Part IV
Clinical Relevance in Disease States- Infection

Aging and HIV Disease: Synergistic Immunological Effects?

Rita B. Effros

Contents

Abstract: The population of HIV-infected adults is progressively aging, due to more effective treatments that lower the viral load. Since aging and HIV disease each have major detrimental effects on the immune system, it is possible that in older persons who are infected with HIV-1, the immune changes due to the infection combined with those that occur with age may synergize to exacerbate the disease. Indeed, clinical studies have already documented older age as an independent risk factor for more rapid HIV disease progression. Moreover, immunological recovery in older individuals treated with antiretroviral drugs is less robust than in younger adults, even with equivalent levels of viral suppression. The challenge to future research will be to develop a detailed mechanistic understanding of the interplay between HIV-related and age-related immunological changes. This information will advance our theoretical understanding of the immune system, and, at the same time provide practical information regarding age-appropriate approaches to therapy and prophylactic vaccines.

1 Introduction

Chronic infection of young individuals with human immunodeficiency virus (HIV-1) is associated with immunological changes reminiscent of those that occur during normal aging. Indeed, HIV disease has even been proposed as a model of premature immunosenescence (Appay and Rowland-Jones 2002a). In young persons infected

R. B. Effros (✉)
Department of Pathology & Laboratory Medicine
David Geffen School of Medicine at UCLA
10833 Le Conte Avenue
Los Angeles, CA 90095-1732
Tel: 310-825-0748
Fax: 310-267-2058
E-mail: reffros@mednet.ucla.edu

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with HIV-1, the pace of immunological change is accelerated. Compounding this effect, the cohort of HIV-infected persons is actually aging chronologically as well. Recent data from the U.S. Centers for Disease control and Prevention indicate that the cumulative number of AIDS cases in the U.S. in persons > 50 years of age quintupled during the last decade, with similar trends reported in Europe (Grabar, Weiss and Costagliola 2006). In New York City, the epicenter of AIDS in the U.S., 30% of HIV-infected persons are over age 50. Aging of the baby boomers, the increased sexual activity of elders in the era of erectile dysfunction drugs, and the prolonged survival of those infected with HIV-1 are among the contributory factors to the overall increase in age of the HIV-1-infected population.

HIV-1 infection and aging each have major effects on the immune system, raising the possibility that in older persons who are infected with HIV-1, the immune changes due to the infection combined with those that occur with age may synergize to exacerbate the disease. Indeed, age is an independent risk factor for more rapid disease progression, and immunological recovery after antiretroviral drug treatment in older individuals is less robust than in younger adults, even with equivalent levels of viral suppression. (Rosenberg, Goedert and Biggar 1994; Darby, Ewart, Giangrande, Spooner and Rizza 1996; Fordyce, Singh, Nash, Gallagher and Forlenza 2002; Egger et al. 2002; Shah and Mildvan 2006). It therefore becomes essential to develop a detailed mechanistic understanding of the interplay between HIV-related and age-related immunological changes. Efforts in this direction may ultimately lead to novel age-appropriate therapies to enhance immune control over the virus. Immune-based approaches to therapy may, in turn, reduce the need for drugs that target the virus. This is important because one of the emerging issues with respect to the elderly is that many of the antiviral therapies are not tolerated well in this group (Casau 2005). Moreover, long term antiretroviral therapy (ART) may interfere with certain medications or exacerbate age-related pathologies.

This chapter will review immune system changes that are common to human aging and HIV disease, highlighting those areas that merit more detailed investigation. One of the fortuitous outcomes emerging from the confluence of research on aging and HIV disease is that the 2 fields are mutually benefiting each other. Indeed, information on T-cell changes that occur during normal aging, many of which are due to untreated persistent infections, has caused HIV biologists to focus on the immune consequences of the chronic antigenic stimulation. Conversely, detailed analysis of immune reconstitution dynamics following ART, which lowers the level of HIV-1, provides immunogerontologists with a unique model system to test the hypothesis that reducing chronic antigenic stimulation retards age-related deleterious changes within the human memory T-cell compartment.

2 Aging and HIV Disease Progression

There is an extensive body of research suggesting that age constitutes a significant risk factor for more rapid disease progression and a strong predictor of increased AIDS-related mortality, both in the presence and absence of ART (Ferro and Salit

1992; Phillips et al. 1991; Kalayjian et al. 2003; Rosenberg et al. 1994; Blatt et al. 1995; Darby et al. 1996; Fordyce et al. 2002; Egger et al. 2002). Moreover, even though virologic efficacy of ART may be equivalent in young and old persons, immunological recovery is, nevertheless, often slower and blunted in older HIV-infected adults (Shah et al. 2006; Manfredi 2004). The negative effect of older age has been observed in persons infected via blood transfusion as well as intravenous drug use. A study on more than 6,000 HIV-infected persons documented that those who were older than age 50 had a significantly increased risk of contracting AIDS wasting syndrome and AIDS dementia, and showed a shortened survival time after AIDS diagnosis (Balslev et al. 1997). Even after adjusting for patterns of complicating diseases, the effect of age persisted. Clearly, a more comprehensive understanding of the effect of age on immune reconstitution within multiple lymphoid compartments is critical in order to develop strategies to prevent the increased incidence/severity of opportunistic infections and the poor responses to vaccines.

Chronic HIV-1 infection is also associated with earlier onset of a number of age-related diseases/pathologies, many of which involve the immune system. Comorbid conditions, such as cardiovascular disease and colon cancer, occur at younger ages in HIV-1-infected patients, an observation that is beginning to affect screening recommendations (Berretta and Tirelli 2006; Engels et al. 2006; Orlando et al. 2006; Palella, Jr. et al. 1998; Murphy et al. 2001; Guy-Grand et al. 1991; Palella, Jr. et al. 2003). Chronic immune activation, a signature feature of HIV-1 disease, is known to contribute to bone loss (Arron J.R. and Choi 2000), which is already accelerated with age. Indeed, one of the immune correlates of hip fracture in a group of uninfected elderly women is the increased proportion of $CD8^{+}CD57^{+}$ T-lymphocytes (Pietschmann et al. 2001). This same cell subset, which has been shown to have telomere lengths consistent with replicative senescence, is significantly increased in HIV-infected persons (Brenchley et al. 2003).

It is well-established that aging is associated with a dramatically increased risk of developing cancer. Indeed, old age carries a cancer risk exceeding that of smoking. The diminished immune surveillance associated with the general immune system deterioration has been assumed to play a significant role in the age-associated cancer risk. Interestingly, chronic HIV infection is also associated with increased cancer incidence, further implicating immune deficiency. A recent meta-analysis compared cancers in HIV-infected with immunosuppressed transplant recipients (Grulich, van Leeuwen, Falster, and Vajdic 2007). Both populations showed increases in cancers with a known infectious cause, such as EBV lymphomas, liver cancer, and human papilloma virus (HPV)-associated cancers, including those of the mouth, penis, anus, liver, stomach, esophagus, larynx and eye. In cancers that are associated with persistent infections, such as EBV, exhaustion of the relevant virus-specific CD8 T-cell response is believed to be one of the contributing factors (Effros 2004). Overall, the similarity in the patterns of increased cancer risk in the elderly and in HIV-infected younger persons is consistent with the notion that the immune deficiency, rather than other risk factors, is responsible for the increased cancer incidence associated with chronic HIV disease. These and other data predict that the combination of aging and HIV disease will further increase the cancer risk,

which would be consistent with the notion of synergy between the immune effects of each separate condition.

In considering the combined effect of HIV and aging on immunosenescence, it should be emphasized that there are two categories of older HIV-infected persons—those who become infected during youth, but survive to old age due to successful treatment, and those individuals who first become infected during old age. Most of the data on aging and HIV are derived from the first category, with minimal information on persons who become infected when they are already old. This latter group of elderly persons may be at a distinct disadvantage, given that the initial control over HIV-1 during the primary infection is so critical in terms of the long term effect on the rate of disease progression. Since aging itself is associated with suboptimal responses to acute infections, from this standpoint alone, the newly infected elderly would be predicted to be at greater risk of more rapid progression to AIDS. A second issue that affects disease progression in newly infected elderly persons relates to the initial diagnosis. It is rare that physicians discuss sexual activity or safe-sex with elderly persons, and even in the face of symptoms suggestive of HIV, blood tests for the virus are rarely advised. Thus, HIV disease may be diagnosed later in older persons, which will have an additional impact on the rate of progression to AIDS.

3 T-lymphocyte Changes During Aging

Changes in cellular immunity are considered to be the main factors responsible for the well-documented increases in infection-related morbidity and mortality in the elderly. CD4 T-lymphocytes are key players in the immune response to pathogens and vaccines, and during aging, the requisite helper functions with respect to both B-lymphocytes and CD8 T-lymphocytes are diminished (Haynes and Swain 2006; Haynes, Eaton and Swain 2002). In addition to the reduced number of recent thymic emigrants, as determined by T-cell excision circle (TREC) analysis (Douek et al. 1998), the naïve CD4 T-lymphocytes that are produced show specific functional decrements. For example, defective T-cell help is responsible for the delay, reduced size, and diminished number of B-cell germinal centers in old mice (Zheng, Han, Takahashi and Kelsoe 1997). Similarly, alterations in CD4 T lymphocyte function have also been implicated in the reduced level of B-cell hypermutation (Yang, Stedra and Cerny 1996) and in the failure to produce high titer antibody in response to influenza vaccination (Swenson and Thorbecke 1997). CD4 T-lymphocytes also provide help for CD8 T-lymphocyte responses, most notably in chronic diseases, and are required for the maintenance of CD8 T-lymphocyte memory after acute infections (Sun, Williams and Bevan 2004). Therefore, the age-associated reduced numbers and quality within the naïve T-cell pool affect multiple facets of immunity.

The progressive reduction of naïve T-lymphocytes with age is due to the combined effects of thymic involution and the homeostatic pressure of the expanded memory T-cell population. The lower numbers of naïve T-cells are associated with

blunted capacity to respond to neoantigens, such as those present in vaccines. The reduced proportion of naïve T-cells also has an impact on cancer, which, as noted above, increases with age and during HIV disease. Interestingly, thymic output is related not only the development of cancer, but also to tumor progression. Specifically, in the most deadly form of brain tumor, glioblastoma multiforme, the number of recent thymic emigrants within the CD8 T-cell subset influences both tumor antigen recognition and age-dependent mortality (Wheeler et al. 2003). Thus, a variety of age-associated defects have been identified for the naïve T-lymphocyte subset, all of which may contribute to the phenomenon of immunosenescence, but arguably to a lesser extent than changes that occur within the memory T-lymphocyte population, as will be discussed below.

Aging in humans is associated with significant changes within the memory CD8 T-lymphocyte compartment, particularly in the cytotoxic T-lymphocyte (CTL) responses to viruses, where both delayed and diminished responses have been documented (Deng, Jing, Campbell and Gravenstein 2004; Po, Gardner, Anaraki, Katsikis and Murasko 2002; Zhang et al. 2002). Within the memory pool of elderly humans, there are clonal expansions of CD8 T-lymphocytes that often occupy a large proportion of "immunological space" and which are also associated with a constriction of the available T-cell repertoire (Ouyang et al. 2003). A large proportion of the lymphocytes within the clonally expanded populations lack expression of the CD28 costimulatory molecule.

Based on extensive cell cultures studies, it appears that the increased proportions of CD28-negative ($CD28^-$)T-lymphocytes in the elderly may be the in vivo correlates of cells that reach the end stage of irreversible cell cycle arrest in vitro following multiple rounds of antigen-driven proliferation. These cells show permanent and irreversible loss of CD28 expression (Effros et al. 1994). Similar to lymphocytes in senescent culture, $CD8^+CD28^-$ T-lymphocytes tested ex vivo are resistant to apoptosis (Spaulding, Guo and Effros 1999; Posnett, Edinger, Manavalan, Irwin and Marodon 1999), show minimal proliferative potential (Effros et al. 1996; Almanzar et al. 2005) and have shortened telomeres (Monteiro, Batliwalla, Ostrer and Gregersen 1996; Effros et al. 1996). CD8 T-lymphocytes that reach replicative senescence in culture also produced high levels of 2 proinflammatory cytokines (TNFα and IL-6) that are associated with a variety of age-related pathologies, and whose concentration is increased in the serum of frail elderly individuals.

The clinical relevance of age-related changes within the T-cell compartment is underscored by data from longitudinal studies in humans, which have identified a cluster of T-cell parameters, the so-called "immune risk phenotype" (IRP) that is predictive of early mortality in the very old. These include an inverted CD4/CD8 ratio, poor proliferative responses and high proportions of $CD8^+CD28^-$ T-lymphocytes. The IRP is significantly associated with latent viral infections, particularly CMV, and to lesser extent with Epstein-Barr virus (EBV) and varicella zoster (Ouyang, Wagner, Wikby, Remarque and Pawelec 2002). Interestingly, immune control over CMV is also relevant with respect to HIV-1 disease: in patients with AIDS, detectible plasma CMV viremia is an independent predictor of death even after adjusting for HIV-1 level and CD4 T-cell counts (Wohl et al. 2005). The above mortality data

from both aging and HIV-1 disease suggest that the continuous antigenic stimulation of CD8 T-cells involved in maintaining the latent status of persistent viruses plays a major role in the accumulation of dysfunctional virus-specific lymphocytes, resulting in the reconfiguration of the aging immune system (Pawelec et al. 2004a).

4 CD8 T-cell Replicative Senescence in HIV Disease

As in most viral infections, HLA Class I-restricted CTL are a critical component of immunological response to HIV-1. The decline in plasma viral RNA after the appearance of HIV-specific CTL during acute infection (Koup 1994; Borrow, Lewicki, Hahn, Shaw and Oldstone 1994) and the prognostic significance of vigorous CTL responses in disease progression (Carmichael, Jin, Sissons and Borysiewicz 1993; Connor, Mohri, Cao and Ho 1993) highlight the key role of CTL. These observations in humans are further bolstered by experiments in rhesus macaques, where depletion of CD8 T-lymphocytes led to striking increases in plasma SIV RNA (Schmitz et al. 1999; Jin et al. 1999). Thus, there is strong indication that CTL are critical in HIV-1 immunopathogenesis, and, it follows that viral persistence and disease progression are due, at least in part, to the eventual failure of CTL.

Similar to aging, chronic infection with HIV-1 is associated with reduced thymic function. In HIV disease, the number of recent thymic emigrants, as determined by TCR excision circle (TREC) analysis of both CD4 and CD8 naïve T-cells, is reduced (Nobile et al. 2004). There is also evidence suggesting that naïve T-cells generated during aging and/or HIV infection may be qualitatively different from those generated during youth. Telomere measurements on 2 populations of naïve CD4 T-lymphocytes, one that represents the most recent thymic emigrants, and the other that has lost expression of CD31 due to homeostatic proliferation (defined by the CD31 marker) show that both types of naïve cells undergo telomere shortening with age. Indeed, the naïve CD4 T-cells in young HIV-infected persons were shown to have telomere lengths that were similar to uninfected persons 30 years their senior (Rickabaugh et al. 2007). These cells also had reduced levels of telomerase activity compared to uninfected controls.

Even the most antiretroviral successful treatment strategy does not eradicate the virus, resulting in ongoing stimulation/replication of HIV-1-specific CD8 T-lymphocytes over many years. Indeed, it is likely that the persistence of suboptimal (i.e., low perforin) HIV-1-specific CD8 T-cell responses despite prolonged therapeutic viral suppression is associated with continuous proliferation and telomere shortening, which can eventually lead to the end stage cell cycle arrest known as replicative senescence. Telomere shortening within the CD8 T-cell subset in HIV-1-infected persons has, in fact, been documented by several investigators (Palmer et al. 1997; Effros et al. 1996; Wolthers et al. 1996). Conversely, robust, continuous proliferation and CTL function of HIV-specific CD8 T-lymphocytes has been identified as a key biomarker of long-term nonprogressors (Migueles et al. 2002).

The importance of telomere maintenance in retarding the process of replicative senescence is underscored by studies demonstrating that gene transduction of HIV-specific CD8 T-lymphocytes from infected donors with the human catalytic component of telomerase leads to indefinite proliferation, increased suppression of viral production by acutely infected CD4 T-lymphocytes, and enhanced HIV-specific IFN-γ secretion, consistent with the importance of telomere length maintenance in anti-viral CTL (Dagarag, Evazyan, Rao and Effros R.B. 2004). Gene transduction with hTERT also retards loss of CD28 expression, which is important, since chronic infection with HIV is associated with increased proportions of CD28- T-cells (Appay et al. 2002b; Effros et al. 1996; Brinchmann et al. 1994).

As with aging, in chronic HIV infection, the presence of CD8 T-cells that are $CD28^-$ is associated with deleterious outcomes. A recent study compared the predictive value of CD28 on CD8 T-cells between two carefully matched HIV-infected cohorts—one that progressed to AIDS within 4 years, and the second that progressed more slowly (i.e., > 8 years). The data show that the fast progressors had significantly greater proportions of $CD8^+CD28^-$ T-lymphocytes at the start of the study (Cao 2007). Moreover, the telomere length of the $CD8^+CD28^-$ T-cells in young (mean age 43) HIV-infected persons is the same as that of PBMC from centenarians (Effros et al. 1996), consistent with the notion that HIV disease may represent premature immunological aging (Appay et al. 2002a). Interestingly, CMV, which plays a key role in aging, is also important in HIV disease. It has been shown that in HIV-infected persons who have progressed to AIDS, detectible plasma CMV DNA was an independent predictor of death even after adjusting for HIV-1 level and CD4 T-cell counts (Wohl et al. 2005).

5 Chronic Antigenic Stimulation and Replicative Senescence

Although the total number of T-cells in the peripheral blood remains stable throughout life in the very healthy elderly (Pawelec et al. 2005), there are marked changes in the relative distribution of T-lymphocyte subsets. In particular, there is a significant decrease in the proportion of naïve CD8 T-lymphocytes, which is accompanied by increased proportions of memory CD8 T-lymphocytes. Most of these memory cells are part of clonal expansions that are specific for persistent viruses, mainly CMV, but also EBV and VZV (Pawelec et al. 2005). Although these viruses do not necessarily reemerge or cause disease, it is becoming increasingly evident that maintaining control over persistent infections over many decades is "costly" in terms of overall immune function (Pawelec et al. 2004a). Thus, it seems that chronic antigenic stimulation of CD8 T-lymphocytes plays a central role in age-related reconfiguration of the human immune system.

In the elderly, replicative senescence within the CD8 T-lymphocyte population is associated with a variety of deleterious clinical outcomes. For example, one of the key immune correlates of reduced vaccine responses is the presence of high proportions of CD8 T-cells that lack CD28 expression. Furthermore, clonal expansions of CD8 T-cells that are CD28- are part of a so-called "immune risk phenotype"

(IRP), which is predictive or early mortality in the very old (Wikby et al. 2002). As mentioned above, the IRP is significantly associated with latent viral infections, particularly CMV. High proportions of senescent CD8 T-lymphocytes are also associated with osteoporotic fractures in older women (Pietschmann et al. 2001), and with accelerated disease progression in the autoimmune disease, ankylosing spondylitis (Schirmer et al. 2002). Finally, in patients with head and neck tumors, the $CD8^{+}CD28^{-}$ T-cell subset undergoes expansion during the period of tumor growth, but is reduced following tumor resection (Tsukishiro, Donnenberg and Whiteside 2003), underscoring the putative role of chronic antigenic stimulation in the generation of senescent CD8 T-cells.

It has been proposed that persistent herpes virus infection may cause CD8 T-lymphocyte replicative senescence in vivo. The persistent nature of these infections is believed to periodically stimulate T-cell responses, resulting in considerable proliferation and clonal expansion of virus-specific CD8 T-cells over time (Appay et al. 2002b). Most of these infections are acquired during youth and establish chronic infection with latency and reactivation, so that by old age there is a cumulative effect of chronic periodic antigenic stimulation of CD8 T-cells causing accumulation of senescent cells (Pawelec et al. 2004b). Chronic infection with CMV seems to be important with respect to HIV disease as well. During the primary (acute) phase of HIV infection, CMV-specific CD8 T-cells in the blood become activated (Doisne et al. 2004), and once the infection becomes chronic, a large proportion of the CD8 T-cell pool is directed at CMV.

The herpesviruses CMV/EBV/VZV establish latency with intermittent reactivation causing chronic intermittent antigenic stimulation leading to replicative senescence. The effect is even more dramatic effect during chronic infection with HIV-1, which persists with exuberant ongoing viral replication and therefore vigorous chronic antigenic stimulation of the CD8 T-lymphocyte pool. This accelerated process of stimulation and senescence would therefore be an ideal model to study, in a short time frame, the aging-associated immune dysfunction caused by ongoing significant antigenic stimulation. HIV-1 provides an additional experimental advantage in that it is a chronic viral infection for which viral replication is easily quantitated and blunted by antiviral treatment. Asymptomatic chronic CMV/EBV/HSV infections, in contrast, are not typically monitored for viral replication or treated due to their predominantly latent state. Thus, studies comparing age-matched treated and untreated HIV-1-infected persons might provide novel insights into the role of chronic antigenic stimulation on the process of replicative senescence.

6 Gut-Associated Lymphoid Tissue (GALT): the Missing Link in Aging Research

In humans, essentially all the information on the immune system has been derived from studies on peripheral blood, which contains approximately 2% of total body lymphocytes. As noted above, a salient finding from those studies is the profound

alteration in function and composition of the memory CD8 T-lymphocyte pool, due, in large part, to the progressive accumulation of cells with features of replicative senescence. There are no data on the age-related changes in CD8 T-lymphocytes in the human gastrointestinal tract, the major reservoir of lymphocytes, and an anatomical region of high antigenic exposure.

The data from animal studies suggest that aging is associated with significant alterations within the GALT, underscoring the need for similar studies in humans. Significantly, changes in the distribution of CD8 T-lymphocytes in the GALT have been observed in aged rats (Daniels, Perez and Schmucker 1993). Mucosal immune system studies in mice have documented age-related reduced frequencies of naïve CD4 T-lymphocytes and dendritic cells in Peyer's patches (Fujihashi and McGhee 2004). Defects in mucosal IgA secretion (Taylor, Daniels and Schmucker 1992) as well as in helper T-cells, CTL function and mucosal vaccine responses have been described for old mice (Fayad, Zhang, Quinn, Huang and Qiao 2004). Finally, the reported age-associated reduction in immune responses to cholera toxin and *E. coli* enterotoxin, which are adjuvants frequently used in mucosally-delivered vaccine preparations, may have broad implications for vaccine success in the elderly (Schmucker, Heyworth, Owen and Daniels 1996). Based on these animal studies, it has been proposed that age-associated alterations arise in the mucosal immune system of the gastrointestinal tract earlier than in the peripheral immune compartment (Koga et al. 2000). These data underscore the need for detailed characterization of the effect of aging on the human GALT.

HIV disease, which, as noted above, shows many immunological parallels with aging, provides a unique opportunity to elucidate changes within the GALT that are due to chronic antigenic stimulation. In fact, it is becoming increasingly recognized that most of the immunological "action" during HIV-1 infection occurs in the gut. Regardless of the route of transmission, the HIV-1 virus selects CD4 T-lymphocytes that also express CCR5 receptors, most of which reside in the gut, with enhanced per-cell CCR5 expression as compared to the blood (Anton et al. 2000). Indeed, treatment strategies based on peripheral blood measurements of CD4 T-lymphocytes or level of viremia have been described as "misguided", since these values are often an underestimate of the profound and continuous loss of CD4 T-lymphocytes in the gut (Veazey and Lackner 2005).

The importance of early and persistent immune responses within the gut mucosa is highlighted in comparisons between long-term nonprogressors and those with high levels of viremia, in which the former show prolonged maintenance of mucosal T-lymphocytes, enhanced virus-specific responses and distinct gene expression profiles (Sankaran et al. 2005). Once the infection has become chronic, the CD8 T-cell response in the gut is "too little, too late", with a magnitude that is <5% of that seen in any other lymphoid organ (Reynolds et al. 2005). Indeed, the ultimate failure of the immune system has been suggested to occur when CD4 and CD8 T-lymphocytes are unable to sustain sufficient frequencies of effectors in both lymphoid and extra-lymphoid tissues, particularly the gut (Grossman, Meier-Schellersheim, Paul and Picker 2006).

There is accumulating evidence that HIV-1 may continue to replicate in mucosal tissues, despite being undetectable in the blood. A recent study, which compared the

viral burden of DNA and RNA in lymphocytes from the gastrointestinal tract to lymphocytes from the blood concluded that the GI mucosal lining carries a disproportionately high viral burden (Comi et al. 2001). In fact, quantifiable levels of HIV-1 can be detected in rectal mucosa-associated tissue despite years of undetectable levels of plasma HIV-1 RNA (Anton et al. 2003). Also, in some women, levels of HIV-RNA are higher in the genital mucosa compared to the blood (Neely et al. 2006).

Peripheral blood studies may also fail to reflect the level of immune reconstitution in the gut. In a seven year study of HIV-1-infected individuals who began ART shortly after infection, it was observed that although the blood population of CD4 T-lymphocytes rebounded to normal levels, a subset of lymphocytes within the gut remained depleted in 70% of the subjects. After three years of intensive drug therapy that suppresses HIV-1 replication very effectively, most patients still had only half the normal number of CD4 effector memory T-lymphocytes in their gastrointestinal tracts (Mehandru et al. 2006). All of these data from studies on HIV disease underscore the need for increased research on the human gut mucosal immune compartment, which has, for various reasons, heretofore been ignored in human immunological studies.

7 Translational Implications

One of the shared features of immunosenescence and AIDS is the accumulation of memory CD8 T-lymphocytes with features of replicative senescence. In both aging and HIV disease, the driving force seems to be chronic antigenic stimulation by persistent viruses. Clearly, prevention of primary infection with these viruses would be the most efficient strategy to prevent replicative senescence. However, it is highly unlikely that prophylactic vaccines against CMV and HIV-1 will be developed in the foreseeable future. Another possible approach is to reduce the antigenic burden by treatments directed against the virus itself. Anti-CMV therapy is usually reserved for situations of extreme immunosuppression, such as in organ transplant patients or the final stages of HIV disease, but it is possible that expanding the criteria for treatment to include all CMV seropositive individuals may lead to improved immune function during aging and AIDS. Antiretroviral therapy (ART) against HIV does, in fact, reduce the antigenic burden, and should theoretically also retard the generation of senescent HIV-specific CD8 T-cells, but no studies have actually addressed this question.

An alternative to reducing the antigenic burden is to augment the function of the virus-specific CD8 T-cells by retarding replicative senescence. For example, since senescent CD8 T-cells no longer express the CD28 costimulatory molecule, one approach that has been used is gene transduction with CD28. Indeed, the reexpression of an intact signaling CD28 molecule in CMV- or HIV-specific CD8 T-cells that had lost CD28 expression led to the restoration of IL-2 production and autocrine-induced proliferation in response to antigen recognition (Topp et al. 2003). Another approach to modulating replicative senescence is based on the enzyme

telomerase, which is upregulated in T-cells during primary and secondary antigenic stimulation, but becomes undetectable by the third and all subsequent stimulations. Transduction of HIV-specific CD8 T-cells isolated from HIV-infected persons with the gene for hTERT (the catalytic telomerase component) results in increased proliferative potential, telomere length stabilization, and enhanced ability to control viral replication (Dagarag et al. 2004; Dagarag, Ng, Lubong, Effros R.B. and Yang 2003). These proof-of-principle demonstrate that telomerase-based immunomodulatory strategies may be practical approaches to enhancing anti-viral CD8 T-cell function in both aging and AIDS. Indeed, preliminary studies show that exposure of CD8 T-cells to certain small molecule telomerase activators leads to increased proliferation and antiviral function (Fauce et al. 2005).

If replicative senescence can be retarded, the result would be a reduction in the proportion of senescent T-cells, and presumably the associated deleterious clinical effects noted above. Thus, more detailed studies on the process of T-lymphocyte replicative senescence may lead to improved prognosis for both aging and HIV disease. An additional benefit of immune-based approaches to therapy may be a reduced need for drugs that target HIV-1. Many of the current drug treatments are associated with metabolic changes normally associated with aging, including lipodystrophy, dyslipidemia and insulin resistacnce, all of which increase the risk of cardiovascular disease (Morse and Kovacs 2006). Thus, HIV disease is associated not only with premature immunosenescence, but also in treatment-associated acceleration in the appearance of many other physiological features of aging (Morse et al. 2006).

8 Concluding Remarks

Treatment advances have resulted in increased life expectancy for persons infected with HIV, which is leading to the "graying" of this cohort (Hinkin, Castellon, Atkinson and Goodkin 2001). In addition, the age of primary infection with HIV-1 is increasing, due to the greater levels of high risk behavior in older adults. The question of whether the immunological changes associated with HIV-1 infection synergize with those that occur during chronological aging has not been addressed. Elucidation of the underlying immune system basis for the relationship between age and HIV-1 disease progression will have far-reaching translational/treatment implications for the progressively increasing elderly population of HIV-1-infected persons. If it turns out that older HIV-infected persons have less immunological reserve, the timing of treatment initiation may require modification. Indeed, many of the current guidelines have been derived from correlations between CD4 T-cell counts and opportunistic infection incidence in younger persons. In addition, since HIV-1 persists with exuberant ongoing viral replication and therefore vigorous chronic antigenic stimulation, particularly of the CD8 T-cell pool, this infection constitutes an ideal model to study the effects of chronic antigenic stimulation on immune dysfunction. It is anticipated that the convergence of immunological studies

in the areas of HIV disease and aging will undoubtedly lead to new paradigms for medical care and vaccine strategies for both situations.

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Role of Immunosenescence in Infections and Sepsis in the Elderly

Tamas Fulop, Steven Castle, Anis Larbi, Carl Fortin, Olivier Lesur and Graham Pawelec

Contents

T. Fulop (✉)
Research Center on Aging, Immunology Program
Geriatric Division, Faculty of Medicine
University of Sherbrooke
Quebec, Canada

S. Castle
Geriatric Research Education
and Clinical Center (GRECC)
VA Greater Los Angeles
Healthcare system, UCLA School of Medicine
11301 Wilshire Boulvard, Los Angeles CA, 90073, USA

A. Larbi · G. Pawelec
Ageing and Tumour Immunology Group
Center for Medical Research, University
of Tübingen Medical School
Tübingen, Germany
Clinical research Center

C. Fortin · O. Lesur
Graduate Immunology Program
Division of Pulmonology
Department of Medicine Faculty of Medicine
University of Sherbrooke

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Abstract: It is well-known that infections and sepsis are increased in elderly subjects, and that the immune system changes with age. The question arises whether dysfunctionality of the immune system, or immunosenescence, contributes to this increased incidence of infections and if so, how. As the immune system evolved to protect against infection, the role of aging is likely to be important for the increased occurrence, progression and outcome of infections and sepsis in the elderly. However, the intricate multiple mechanisms that contribute to this increase are difficult to dissect with certitude and remain controversial. Immune alterations most likely to contribute to this overwhelming clinical burden of infections and sepsis will be reviewed in this chapter.

1 Introduction

It is well-known that infections and sepsis are increased in elderly subjects and that the immune system becomes in many ways dysfunctional with aging [1–3]. The question arises whether immunosenescence contributes to this increased incidence of infections and if so, how. The answer would seem to be a priori in the affirmative, but it is very difficult to ascertain a direct relation between these two phenomena. Immunosenescence differentially affects the various components of the immune system; moreover, many extrinsic factors such as nutrition, chronic diseases, chronic antigenic stress or hormonal changes also contribute to immunosenescence [4–7]. Furthermore, aging is associated with a low grade inflammatory state that might be causally involved in inappropriate responses to infection.

2 Infections in the Elderly

Typical bacterial infections including pneumonia, urinary tract, and skin and gastrointestinal infections are a common problem in older adults [2]. Not only bacterial, but viral infections such as respiratory tract infections due to influenza A or Respiratory Syncytial Virus, or reactivation of Herpes zoster are also very common in elderly. Moreover, pseudomembraneous colitis related to microbial colonization of Clostridium difficile or methicillin-resistant Staphylococcus aureus (MRSA) in severely ill patients treated with antibiotics is becoming an important health issue in elderly people [2]. One of the most significant public health problems is Influenza virus infection, which causes 10,000–40,000 excess deaths in the USA, of which 90% are in persons over 65 years [8]. Influenza is the fifth leading cause of death among people aged 50 and older and this is a major target of vaccination campaigns [9–11]. The incidence of pneumococcal infections increases dramatically in people over 75 years of age [12]. Mortality is higher in the elderly and rises with increasing age, approaching almost 80% in those over 85 years of age. Rates of bacteremia and meningitis from pneumococcal

infections are also higher in the elderly [6]. The incidence of Herpes zoster is also greater in people over 75 years of age [13]. Not only the incidence and prevalence of infections are increased in the elderly but also the consequences and the burden in terms of morbidity and mortality.

The problem of infection is even greater in elderly nursing home residents, who are particularly vulnerable to infections. In addition to decreased immune responsiveness, malnutrition and chronic diseases, long-term care facilities themselves provide environments that promote infectious outbreaks [3]. Elderly people in nursing homes suffer infections due to urinary catheters more often and have more frequent oropharyngeal colonization with Gram-negative bacilli [14, 15]. Nearly one third of persons 80 years of age and older live in nursing homes where antibiotic resistance is a growing problem [3], and residents who are infected are at a higher risk of mortality.

3 Sepsis in the Elderly

Sepsis is defined as the systemic host response to infection [16, 17]. Most of the time it appears as a life-threatening clinical situation. It is not a single disease but is an intricate and heterogeneous process expressed through the interaction of a complex network of biochemical and cellular mediators and amplification cascades. Its severity is mainly determined by the causative agent, the patient's genetic background and the rapidity of medical intervention [17]. This inherently complex process, reflecting the dynamic interaction of an acute, life threatening infection with the adaptive protective mechanisms of the host and its environment, is frequently modified often in an unpredictable manner by the effects of advancing age, sex and/or acute and chronic underlying disorders [16].

Each year in the USA nearly 2500 cases of sepsis occur per 100,000 persons aged 85 years, with elderly persons being much more likely to suffer sepsis and bacteremia than younger subjects [3,18]. Incidence rates of sepsis increased 20.4% faster among elderly persons than among younger persons from 1979 to 2002 (mean increase per year, 11.5% vs. 9.5% $p<0.001$). Other large studies have reported that the incidence of sepsis and bacteremia increase with older age [19, 20]. Furthermore, the microbiology of these infectious diseases is also different in the young and old [3]. In contrast to young sepsis patients, most of these disorders in the elderly are due to Gram-negative organisms. Escherichia coli was found to be the responsible agent in most cases in the elderly, while in young subjects Staphylococcus aureus was the main pathogen in community-acquired bacteremia [21]. The causative agent tends to be different in nosocomial bacteraemia, in that in the elderly the most frequent pathogens is MRSA while in young patients this is again S. aureus. These differences in the microbiology of sepsis with age are partly explained by the source of infection leading to sepsis among the elderly. Urinary tract infections due to Gram-negative bacteria are more frequently the source of bacteremia or sepsis in elderly than in young patients. In a study of community-acquired bacteremia, Lark et al. [22] found that patients 65 years of age were more likely than younger patients to

have the urinary tract as the source of infection. Other studies demonstrated similar origins for sepsis in aging. These data suggest that the elderly could be more susceptible to Gram-negative bacterial infections than young subjects due at least partly to changes in immune functions with aging. Together, these data show that older age is independently associated with an increased likelihood of severe sepsis although the relationship was not shown to be linear [16].

4 Alterations in the Immune System with Aging which Could Favour the Increase of Infections and Sepsis

4.1 Innate Immune System

The innate immune system includes neutrophils (PMN), macrophages and NK-cells. These cells are the first to encounter any type of infection, whether bacteria or viruses [23]. They recognize pathogens by means of their nonpolymorphic conserved pattern recognition receptors (PRRs) and discriminate between invaders representing a danger for the organism and those not pathogenic [24–26]. One of the most studied groups of PRRs belong to the Toll-like receptor family (TLRs). There are more than 11 members of this family, including TLR4 reacting to Gram-negative bacteria, TLR2 reacting to Gram-positive bacteria and TLR3 and TLR9 reacting to viruses. Stimulation of innate immune system cells via TLRs initiates a complex signal transduction cascade which can result in proinflammatory activation through translocation of NF-kB to the nucleus [24]. This renders the cells of the innate immune system more potent in their effector functions, such as phagocytosis, free radical production, and intracellular killing which result in the destruction of the invader. Such activation may also initiate and modulate adaptive immune responses either by antigen presentation or secretion of different cytokines and chemokines, perhaps as a mechanism required if the innate immune response fails to clear the pathogen.

Ageing affects components of the innate immune system differentially. Some functions are well-preserved, such as phagocytosis, while others are decreased, such as chemotaxis, intracellular killing and free radical production [23]. Furthermore, even if the number of TLRs expressed on the cell surface appears unchanged, their signalling is altered, leading to dysregulated intracellular activation of proinflammatory cytokines [23]. Furthermore, the persistence of infections due to failure to clear the pathogen may result in persistent, chronic, activation.

4.2 Adaptive Immune System

The adaptive immune system responds specifically to a unique antigen via antigenic presentation. The response is either humoral via B-lymphocyte antibody production or cellular via T-cell activation. The T-cell compartment is divided into

helper (CD4+) and effector subsets (mainly CD8+). B- and T-cells also responding to antigens via specific cell surface receptors which initiate a signalling cascade leading to their activation [27]. There could also be chronic antigenic activation in this compartment mainly by latent viruses such as CMV or herpes zoster [28–30]. Furthermore, a network of cytokines plays a major role in orchestrating a coordinated adaptive immune response via T- and B- cells.

T-cell functions are the most altered with age [1, 2, 6]. Following antigenic stimulation, the clonal expansion of T-cells is decreased with age due to altered IL-2 production. There is also a shift from naïve T-cells towards memory T-cells [31]. This shift is partly explained by the involution of the thymus, leading to decreased output of naïve (virgin) T-lymphocytes. One other very important factor seems to be chronic CMV infection, as mentioned above. This leads to the oligoclonal expansion of CD8+ T-cells in the elderly, the accumulation of which, in the form of apoptosis-resistant anergic effector CD8+ memory T-cells, may have far-reaching consequences. These cells may fill the immune space and even suppress the function of the remaining naïve CD4+ T-cells [32–34]. This leads to decreased recognition of novel antigens and in consequence a decreased ability to respond to previously unencountered pathogens. Another alteration, limiting the response of T-cells to stimulation, is altered intracellular signalling following ligation of the TCR and CD28 coreceptor [35, 36]. All these alterations lead to a dysregulated adaptive immune response with aging. Evidence for the clinical importance of viral persistence along with other immune parameters has been provided from longitudinal studies of subjects above 85 years, where it was observed that the increased anti-CMV Ig levels correlated negatively with survival [28–30]. Moreover, these subjects had a lower response to vaccination.

4.3 Low-Grade Inflammation: Inflamm-Aging

An apparent disequilibrium between the relatively reactive innate immune response and the altered adaptive immune response with aging leads to the presence of a low grade inflammatory status with aging [37]. The cause of this low grade inflammation is multifactorial. One of the most important is chronic antigenic stimulation. The antigen can be exogenous, such as bacteria or viruses, or endogenous like the various posttranslationally-modified macromolecules such as DNA or proteins. They can be modified by oxidation, by acylation or by glycosylation. Such altered molecules can stimulate the innate immune response, mainly macrophages via TLRs, thus contributing to sustaining a proinflammatory state [24]. This is measurable in some circumstances as increased circulating levels of IL-6, IL-1β or TNFα. These modifications may also result in the stimulation of adaptive immune responses, recognized in an extreme form by an inverted CD4:CD8 ratio, caused by an overwhelming expansion of CD8+ cells [38]. All these changes contribute to a decreasingly effective immune environment which seems not to be able to respond appropriately to new infectious agents.

5 How Can Immunosenescence Contribute to Increased Infections and Sepsis in the Elderly?

Because underlying disorders are more frequent in the elderly, the role of age is crucial in delineating the true influence of underlying disorders on the host response and susceptibility to infections (Table 1 and 2). Many large epidemiological studies now demonstrate that age is related to the occurrence [39, 40] and prognosis of infection.

5.1 Contribution of the Intestinal Mucosal Defense

The barrier functions of the mucosal components including sIgA, mucins, defensins, gastric acid, and epithelial integrity may be seriously compromized with aging [24].

Table 1 The most significant functional alterations of the immune system with aging potentially implicated in the increased infections and sepsis

Innate immune response	neutrophils and monocytes/macrophages
	↓ ROS production
	↓ intracellular killing
	↓ TLR signalling
	dendritic cells
	↓ antigen presentation
	NK cells
	↓ decreased effector functions
Adaptive immune response	T cell antigenic response:
	↓ proliferation
	↓ Th1 response: IL-2, IFNγ
	↑ Th2 response IL-4, IL-5, IL-10, IL-12
	↓ Delayed type hypersensitivity
	↓ T cells inducers of suppression
	↓ T cell repertoire
	↓ Signal transduction: early, intermediate and late events
	T cell subpopulations
	↓ naive T cells
	↑ memory T cells
	T cell apoptosis:
	↑ CD4+ T cell apoptosis
	↓ T cell repertoire CD8+ T cell apoptosis
	B cell antigenic response
	↓ B cell repertoire
	↓ Amtibody quality ?
Low grade inflammation	Cytokines
	↑ Pro-inflammatory cytokines : IL-6, TNF α
	↑ Anti-inflmmatory cytokines: IL-10

Table 2 The most significant external factors with aging potentially implicated in the increased infections and sepsis

Malnutrition :	Macronutrients
	↑ Lipids
	↑ Carbohydrates
	↓ Proteins
	Micronutrients
	↓ Zinc, Selenium
	↓ Vitamins: Vitamin E, Vitamin C
	↓ Antioxidants
Chronic diseases:	Diabetes mellitus type 2
	Cardiovascular diseases: congestive heart failure
	Dementia
	Autoimmune diseases
	Pulmonary diseases: COPD
	Cerebrovascular diseases
	Cancers
Frailty:	Low grade inflammation
	↑ Pro-inflammatory cytokines : IL-6, TNFa
Chronic antigenic stress:	Chronic infections: CMV, EBV, Herpes zoster
Neuroendocrine changes:	↑ cortisol
	↓ DHEA, Growth hormone

The result of these alterations is that this first line of defense is no longer very efficient at excluding extracellular pathogens or sustaining protective commensals, which can even become pathological. However, there are still very few data regarding how mucosal immunity changes with age and influences the incidence of infections.

5.2 Contribution of the Innate Immune Response

Alterations in the innate immune response with aging as discussed earlier may greatly contribute to the increased incidence of infections and sepsis with aging. Decreased chemotaxis in response to chemokines results in poorer accumulation of the cells necessary for first line defence, including PMN [23]. Lower production of reactive oxygen species by PMN and macrophages detracts from the clearance of pathogens. The presentation of antigens by dendritic cells seems to be fairly well-maintained in healthy elderly, but there may be subtle differences, as well as the speed of processing being decreased [2, 8]. Elderly nursing home patients with significant chronic illness however, do have impaired APC functions [8]. The exact role of NK-cells in the increased infections seen with aging is still controversial, as their exact functional changes with aging have not been determined exactly.

The decreased functions of PMN are particularly important in this setting. These cells are the first line of defense against infection. There is a complex process prior to PMN arrival at the site of invasion, including rolling, adherence, diapedesis and chemotaxis [41]. This process is relatively well-conserved in ageing, although chemotaxis may be compromized. Most importantly, the production of reactive oxygen species (ROS), playing a crucial role in intra and extracellular killing, is altered. It was shown that various Gram-positive bacteria ingested by PMN were not to be destroyed as efficiently as in young subjects [42, 43]. Together, these data reinforce the notion of an important contribution of altered innate immune responses to the increased incidence of infections with aging.

As part of innate immunity, the proinflammatory response to infection which is not diminished in the elderly, may contribute to the increased proinflammatory state commonly observed [16]. However, it should be mentioned that in SENIEUR elderly subjects, selected for exceptionally good health, this low grade inflammation is practically nonexistent [1]. This state of "inflamm-Aging" as it has been dubbed seems to manifest by an increase in the IL-6 level, which may be a reliable marker for functional disability and a predictor of disability and mortality in the elderly [44, 45]. Indeed Cohen et al. [46] have reported that activation of the coagulation (D-dimer) and the inflammatory (IL-6) pathway at baseline is associated with mortality and decline in function. Aging is also associated with inadequate response to infections and sepsis-related stress. Monocytes from elderly patients undergoing surgery produced more TNFα than those from younger patients [47]. After challenge with LPS in healthy young and elderly volunteers the latter showed more prolonged fever response than in younger controls and levels of TNFα and soluble TNF receptor I levels were higher in the elderly [48]. This study suggests that aging is associated with an altered host response with initial hyperreactivity and a sustained secondary antiinflammatory response. Elderly persons with pneumococcal infections also show prolonged and exaggerated cytokine responses, compared with those of younger persons [49]. Higher levels of proinflammatory cytokines, such as TNFα and IL-6, have been observed in elderly patients with sepsis when compared to young subjects [50]. In sepsis these cytokines and others generated in response to toxic microbial stimuli activate leukocytes, promote leukocyte-endothelium adhesion and induce endothelial damage [51].

5.3 Contribution of the Adaptive Immune Response

Aging is associated with dysfunction of T-cell mediated adaptive immunity. Thymic involution together with chronic antigenic stimulation decreases the number and repertoire of naïve T-cells which leads to an inability to respond appropriately to a new antigen. This is correlated with an expansion of memory CD8+ T-cells which fill the "immune space" due to their resistance to apoptosis. Moreover, they may suppress the retained CD4+ T cell response. It is also well-recognized that the B cell-mediated humoral response is also decreased, contributing to the increase of infections by

reduced specific antibody production, by reduced affinity and shrinkage of the B-cell repertoire. As well as B-cell intrinsic changes, decreased CD4+ T-cell help contributes, to these alterations [1]. Furthermore, it was also recently shown that increasing age has a significant impact on the memory CD8+ T-cell response to respiratory virus infections [33]. There is a significant loss of effector memory cells from peripheral sites over time which may reduce the immediate response of memory T-cells to secondary challenge. However, this is efficiently counteracted in part by the long term maintenance of large numbers of memory CD8+ T-cells in the secondary lymphoid organs and the progressively increasing capacity of these cells to generate proliferative recall responses [52]. Overall it appears that T-cell memory is not only maintained for long periods of time, but may also be enhanced in the face of an age-related decline in the capacity of the immune system to respond to new pathogens [33, 53].

5.4 Contribution of the Low Grade Inflammation

The low grade inflammation is only the common pathway of immunosenescence leading to its increased clinical consequences. This state favours the development and progression of other age-associated chronic diseases, such as atherosclerosis, neurodegeneration (dementia), Type 2 diabetes, metabolic syndrome and congestive heart failure [24, 37]. It is well-recognized that these diseases contribute to the further deterioration of defense mechanisms and thus patients suffering from chronic diseases are more susceptible to infections such as influenza or pneumonia. Hence the severity of infectious diseases is greater in patients with chronic underlying disorders compared to healthy elderly subjects. The presence of one or 2 chronic illnesses such as emphysema, diabetes, cardiovascular diseases, chronic renal or hepatic failure, is associated with a 40- to 150-fold increase in the basal incidence rate of influenza pneumonia [4, 6, 54]. Hospital mortality is also related to severity of the underlying chronic diseases, including cardiovascular insufficiency, chronic obstructive pulmonary disease and kidney failure. This is even more striking in nursing home settings.

Furthermore, low grade inflammation plays a specific role in metabolic disorders of the elderly. The production of proinflammatory cytokines affects insulin resistance and muscle wasting (sarcopenia). This leads to a dramatic increase of diabetes in elderly subjects even if they are not obese. Moreover, this proinflammatory state contributes to the appearance of frailty as a newly recognized physiological syndrome [55]. Most of the metabolic alterations related to low grade inflammation can be also found in frailty. Thus, frailty seems to be in a continuum with aging before the development of specific diseases. It is also known that these frail individuals are more prone to infections than those not suffering from this syndrome. The low grade inflammation, metabolic alterations, malnutrition, chronic diseases and sarcopenia all mediate a concerted effect to the increase of infections in the elderly.

The low grade inflammatory status can even have a paradoxical effect in favoring the development of infections. “Overstimulation” might induce a compensatory anti-inflammatory overreaction (e.g., IL-10, IL-13) which could further impair immune

responsiveness toward new antigens. This could also lead to the development of the well-known autoimmune disorders associated with aging.

6 Response to Vaccination

To date approximately 26 different infectious diseases can be prevented by vaccination, influenza being one of them [56]. However, as a result of age-associated immune alterations, the elderly generally have a poorer response to vaccination than the young [10, 11, 57, 58]. This correlates with decreased levels of protective antibodies following influenza vaccination [6]. Cell-mediated immunity represented by the CTL response, which may be even more important for protection than antibody, is decreased too [59]. Not only is the vaccine response impaired in the elderly, but even when it seems adequate, protection from infection is still less than in young subjects. This is probably related to the quality of antibody formed in terms of viral neutralizing. Nevertheless, despite this low efficacy of the immune response, it should be emphasized that vaccination in elderly subjects is efficacious in reducing adverse events [8, 10, 59].

An underlying chronic illness or frailty dramatically increases the risk of influenza infection as well as impairing the response to vaccination. One study on vaccine responses in nursing home residents demonstrated that only 50% of vaccines generated an adequate response based on the definition of a 4-fold increase in antibody titers [54]. However, this population is one of the most targeted for vaccination, taking into account the clinical efficacy of vaccination in elderly subjects [59].

7 Conclusions

Immune dysfunction, mainly in the T-cell compartment, is associated with age even in the healthiest elderly. The cause of this dysregulation is certainly multifactorial. The results of these alterations are obvious in certain clinical situations such as infections. The role of aging is important for the increased occurrence, progression and outcome of infections and sepsis in the elderly. However, the intricate multiple mechanisms that contribute to this increase are difficult to dissect with certitude and remain controversial. Nevertheless, the clinical burden most likely resulting from such immune dysregulation is overwhelming. Strategies should be developed in order to modulate the immune response in such a way that morbidity and mortality caused by infectious disease in the elderly is decreased. More effective vaccination strategies must be developed. Other solutions should be also rapidly sought and implemented to improve the quality of life of the elderly in the rapidly increasing elderly populations of the developed countries.

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Beneficial and Detrimental Manifestations of Age on CD8+ T-Cell Memory to Respiratory Pathogens

Jacob E. Kohlmeier, Kenneth H. Ely, Alan D. Roberts, Eric J. Yager, Marcia A. Blackman and David L. Woodland

Contents

Abstract: Increasing age is associated with a decline in adaptive immunity and poorer responses to vaccination. While specific immune defects have clearly been defined in the naïve T-cell pool of aged individuals, much less is known about the memory T-cell pool. Current data suggest that T-cell memory generated in an aged individual has a reduced capacity to mediate recall responses due primarily to defects in the proliferative capacity of individual cells. These defective recall responses in the aged can be further compounded by the development of 'holes' in the T-cell repertoire due to a dwindling supply of naïve T-cell precursors. In contrast, T-cell memory generated in young individuals undergoes a variety of changes over time including both an increase in the proliferative capacity of individual memory T-cells and a decrease in the overall efficacy of the recall response in the lung. Furthermore, the development of T-cell clonal expansions with age can have a dramatic impact on the makeup of the memory T-cell pool, thereby influencing the number of pathogen-specific T-cells capable of participating in the recall response. Collectively, these changes appear to reflect the redistribution of memory T-cell subsets within the memory T-cell pool and the dysregulation of memory T-cell homeostasis over time. This review outlines each of these processes and discusses their implications for vaccination in the elderly.

D. L. Woodland (✉) · J. E. Kohlmeier · K. H. Ely · A. D. Roberts · E. J. Yager · M. A. Blackman
Trudeau Institute
154 Algonquin Avenue
Saranac Lake, NY 12983
Tel.:(518) 891 3080
Fax: (518) 891 5126
E-mail: dwoodland@trudeauinstitute.org

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1 Introduction

Influenza and other respiratory virus infections are a major human health problem in the United States (Murphy and Webster 1996). They represent a major cause of illness and death in the U.S. and are responsible for an average of approximately 20,000 deaths and 110,000 hospitalizations each year (Glezen 1982). Furthermore, mortality from this class of infections has increased significantly over the last two decades, particularly in the elderly (Pinner et al. 1996). In addition, newly emerging respiratory viruses, such as the highly virulent influenza H5N1 variant that appeared in Hong Kong in 1997, are of particular concern (Gubareva et al. 1998; Shortridge et al. 1998; Subbarao et al. 1998). Thus, there is an urgent need to understand pulmonary immunity to these viruses, especially in the elderly.

Influenza virus infections appear as yearly epidemics that peak in the winter months and are characterized by short incubation periods, high infection rates, and rapid spread through the population (Murphy and Webster 1996). The capacity of influenza virus to generate yearly epidemics depends on the virus' ability to evade humoral immunity. This is achieved in the case of influenza by both antigenic drift due to mutation of coat proteins (which are targets for neutralizing antibodies) and antigenic shift due to reassortment of viral RNA segments. Given the capacity of the virus to evade humoral immunity, cellular immunity plays a major role in controlling secondary virus infections (Rimmelzwaan and Osterhaus 1995; Yewdell et al. 1985). It has been noted that the severity of influenza virus infections in individuals decreases over time and it is believed that this reflects repeated boosting of cross-reactive T-cells (Bender and Small 1993; Frank et al. 1983; Liang et al. 1994; McMichael 1994; Schulman 1970; Sonoguchi et al. 1985). However, beyond a certain age, individuals become more susceptible, suggesting a waning of this memory response (Bender and Small 1993; Bender et al. 1991; Liang et al. 1994). Also the capacity of vaccines to induce cellular memory appears to wane with age (Fagiolo et al. 1993; Murasko et al. 2002). This is a significant problem for public health organizations since the elderly are the most at-risk population for influenza-associated mortality (Belshe 1998). Clearly, there is an urgent need to better understand the mechanisms that underlie the loss of immune function with age and to use this information for the development of approaches that promote improved vaccine efficacy in the elderly.

2 Characteristics of CD8$^+$ T-Cell Memory to Respiratory Pathogens

An important aspect of the adaptive immune system is the capacity to mediate stronger and more effective responses to secondary pathogen challenge as compared to primary pathogen challenge (Dutton et al. 1998; Woodland 2003). This characteristic is referred to as immunologic memory and is mediated by both the T and B cell arms of the immune system. In the case of CD8$^+$ T-cells, increased antigen-specific cell numbers, higher activation status, reduced stimulatory requirements, more rapid induction of effector functions, and altered homing patterns of memory T-cells all contribute to enhanced recall responses (Dutton et al. 1998; Hammarlund et al. 2003; Seder and Ahmed 2003). Memory CD8$^+$ T-cells are also characterized by the capacity to persist for many years after their initial generation and potentially maintain functional immune memory for the life of the individual. While various aspects of T-cell memory have been extensively studied, relatively little attention has been paid to the impact of age on memory T-cell generation and maintenance.

Over the last few years, a great deal of progress has been made in understanding how T-cell memory operates in a complex mucosal organ such as the lung. It is now recognized that memory CD8$^+$ T-cells are heterogeneous in terms of their phenotype and anatomical distribution. This has led to a broad categorization of memory T-cells into central memory cells (T_{CM}) that persist in the secondary lymphoid organs and peripheral or effector memory cells (T_{EM}) that persist in nonlymphoid sites (Cauley et al. 2002; Flynn et al. 1998; Hogan et al. 2001; Lefrancois and Masopust 2002; Marshall et al. 2001; Masopust et al. 2001; Murali-Krishna et al. 1998; Reinhardt et al. 2001; Sallusto et al. 1999; Usherwood et al. 1999). The CD62L (the lymph node homing-receptor) and CCR7 molecules are particularly relevant to this classification since they divide memory T-cells into peripheral (T_{EM}, CD62L$^-$/CCR7$^-$) and systemic (T_{CM}, CD62L$^+$/CCR7$^+$) subsets. Interestingly, most memory CD8$^+$ T-cells generated by an intranasal influenza or Sendai virus infection are of a T_{EM} phenotype (CD62L$^-$/CCR7$^-$).

The anatomical distribution of peripheral and systemic memory CD8$^+$ T-cells predicts that they will be differentially involved in recall responses to secondary viral challenge in the lung. Indeed, it has now emerged that the recall response can be divided into at least three temporally distinct phases, each mediated by different subsets of memory CD8$^+$ T-cells. The first phase involves antigen-specific T_{EM} residing in the lung airways. These cells are the first to encounter the pathogen and they have been shown to mediate early control of a secondary respiratory virus challenge (Hogan, Usherwood et al. 2001; Hogan, Zhong et al. 2001). The second phase involves circulating T_{EM} that are directly recruited to the lung airways from the circulation by inflammatory signals. The recruitment of these cells does not require cognate antigen stimulation and these cells do not divide prior to migration into the lung airways. The third phase involves both T_{EM} and T_{CM} that proliferate in response to antigen and are recruited to the lung airways as fully activated effector

T-cells (Sallusto et al. 1999). Since these cells are proliferating in the secondary lymphoid organs, this phase of the response is sustained through the protracted production of new effector cells (Berenzon et al. 2003; Bjorkdahl et al. 2003; Harris et al. 2002; Hengel et al. 2003; Unsoeld et al. 2002). The combination of the three phases results in a sustained recall response to the infection, and the early control of pathogen load during the first two phases of the response provides time for the proliferative third phase of the recall response to develop and begin producing secondary effector T-cells.

An interesting feature of memory $CD8^+$ T-cells is that they exhibit considerable phenotypic heterogeneity. This heterogeneity defines a number of subpopulations that exhibit distinct trafficking and functional properties. For example, antigen-specific populations of T_{EM} and T_{CM} cells can be further divided into several different subpopulations based on the expression of markers such as CD43, CD27, CD127 and Ly6C (Oehen and Brduscha-Riem 1998; Sprent 1997). It should be emphasized that cells within these subpopulations appear to be resting, despite their expression of markers associated with activation. Recently, we have analyzed the phenotype of memory $CD8^+$ T-cells generated by intranasal Sendai virus infection with a particular emphasis on the expression of markers that distinguish quiescent from semiactivated memory T-cell subsets, such as CD27, CD43 (activated isoform), and CD127 (Baars et al. 2005; Croft 2003; Jones et al. 1994; Kaech et al. 2003; Onami et al. 2002). These studies showed that the memory T-cell pool can be further divided into three distinct subpopulations; $CD27^{hi}/CD43^{lo}$ (most quiescent), $CD27^{hi}/CD43^{hi}$, and $CD27^{lo}/CD43^{lo}$ (most activated; Hikono et al. 2007). Interestingly, these subpopulations differed in their capacity to mediate recall responses to respiratory virus infection in vivo, with the strongest response being associated with the most quiescent phenotype ($CD27^{hi}/CD43^{lo}$). Furthermore, the cells that mediated the weakest recall response ($CD27^{lo}/CD43^{lo}$) also express KLRG1, which has been associated with cell senescence (Voehringer et al. 2001). Thus, these data define a new categorization of memory T-cells that is distinct from the T_{EM} and T_{CM} subsets. As we will discuss below, aging has a significant impact on the distribution of these different memory $CD8^+$ T-cell subpopulations and consequently on immune responsiveness.

3 Generation of T-Cell Memory in the Aged

As a better understanding of T-cell memory has emerged over the last few years, there has been a growing interest in the impact of age on the generation of T-cell memory (Ely et al. 2007; Gupta et al. 2004; Linton et al. 2005). Several studies have clearly established that memory generated in aged individuals is inferior to that generated in young individuals (Haynes et al. 2005; LeMaoult et al. 2000; Miller 1996; Wick et al. 2000). However, the underlying mechanisms for this observation are not clear. The defect does not appear to lie in the capacity of aged individuals to generate peripheral or mucosal memory cells since these cells are generated in large numbers and appear to be functionally normal. Rather, it appears that the

problem lies in the capacity of memory $CD8^+$ T-cells in the aged to mediate the proliferative aspect (phase 3) of the recall responses. Indeed, several studies have shown that memory $CD8^+$ T-cells generated in aged mice exhibit a poor capacity to expand. A critical question is whether this reflects the poor proliferative capacity of antigen-specific $CD8^+$ themselves (an intrinsic effect), or whether it is controlled by the aged environment in which the cells are responding (an extrinsic effect). We have addressed this question using a dual adoptive-transfer system in which we are able to directly compare the proliferative capacities of Sendai virus-specific $CD8^+$ memory T-cells that had been generated in either young or aged mice. These data showed that memory cells generated in aged mice were two to threefold less efficient on a per cell basis than memory cells generated in young mice at mediating recall responses in the lungs and other tissues (Roberts et al. 2005). Since the cells were compared in the same young recipient animals, the data cannot be explained by differences in the viral load, antigen load, degree of inflammation, or other factors thought to be abnormal in an aged environment. Thus, these studies demonstrated that the poor responsiveness of memory $CD8^+$ T-cells generated in aged mice is an intrinsic feature of the cells. One possible explanation for this decreased responsiveness is that the distribution of memory T-cell subpopulations is different in aged mice. Indeed, preliminary evidence suggests that de novo immune responses in aged mice generated a greater number of nonresponsive antigen-specific memory T-cells compared to young mice (Hikono et al. 2007). An alternative possibility is that the characteristics of the $CD8^+$ memory T-cell pool may depend on the quality of T-cell help present during the infection. In this regard, the decline in naïve $CD4^+$ T-cell responses in aged individuals may have a secondary effect on the quality of $CD8^+$ T-cell memory generated (Haynes 2005; Kovaiou and Grubeck-Loebenstein 2006). We are currently investigating these ideas in more detail.

4 Repertoire Loss Associated with Aging Impacts Immunity

A well-characterized consequence of aging is loss of T-cell repertoire diversity. Fewer new T-cells emerge from the thymus, due to age-related involution (Miller 1991; Sempowski et al. 2002). In addition, the proportion of cells with a naïve phenotype ($CD44^{low}$) compared with those of a memory phenotype ($CD44^{high}$) gradually decreases with age, such that in an 18-month-old mouse naïve T-cells constitute less than 5% of total peripheral $CD8^+$ T-cells (Effros et al. 2003; Lerner et al. 1989; Naylor et al. 2005). Finally, there is the development of large clonal expansions that can further reduce repertoire diversity (Callahan et al. 1993; Hingorani et al. 1993; Posnett et al. 1994; Schwab et al. 1997). Thus, there is a dramatic age-associated reduction in repertoire diversity of naïve $CD8^+$ T-cells which is thought to contribute to the well-characterized inability of the elderly to mount effective immune responses to newly encountered antigens (Fagnoni et al. 2000; P. J. Linton and Dorshkind 2004; Messaoudi et al. 2004; Miller 1996; Mosley et al. 1998; Naylor et al. 2005; Nikolich-Zugich 2005). We have previously proposed that consequences

of the declining diversity of the aged naïve repertoire are twofold (Woodland and Blackman 2006). First, we predicted that the decline in numbers of antigen-specific naïve T-cells could drop below a necessary threshold, resulting in the development of 'holes in the repertoire', particularly for those clonotypes with a low precursor frequency. Second, we predicted that the response to new antigens would be increasingly dominated by fortuitously cross-reactive memory T-cells. T-cell recognition has been shown to be highly degenerate (Mason 1998; Nikolich-Zugich et al. 2004), and a great deal of cross-reactivity to viral antigens that are not obviously structurally related has been demonstrated (Selin et al. 2004; Selin and Welsh 2004). In addition, memory T-cells are more readily activated than naïve T-cells (Ahmed and Gray 1996; Dutton et al. 1999; Seder and Ahmed 2003). Thus, as the number of naïve T-cells declines, responses to newly encountered antigens will be increasingly dependent on preexisting cross-reactive memory T-cells. This would result in weaker, highly stochastic responses reflective of an individual's prior antigenic experience.

We have recently obtained experimental evidence for our first prediction by taking advantage of the well-characterized mouse model of influenza virus infection (Yager et al. 2008). Three immunodominant epitopes in C57BL/6 mice have been described: $NP_{366–374}D^b$ (NP), $PA_{224–233}D^b$ (PA) and $PB1_{703–711}K^b$ (PB1; Belz et al. 2000; Deckhut et al. 1993; Townsend et al. 1986; Zhong and Reinherz 2004). Despite the fact that the response to these three epitopes following influenza virus infection of young mice is relatively equi-dominant, we found that the naïve T-cell precursor frequency for these three epitopes was approximately 1:10:30, respectively. We then examined the $CD8^+$ T-cell response elicited in aged mice following de novo infection with influenza virus. Our analysis of the ability of aged mice to clear influenza virus confirmed the reports of others (Bender et al. 1991; Effros and Walford 1983; Po, Gardner et al. 2002) in that clearance was delayed in aged mice. However, we observed a great deal of variation in the ability of individual aged mice to clear virus effectively. This variation could not be explained by a global defect in T-cell responses in individual aged mice, as the frequencies and number of CD8 T-cells in the BAL and lung tissue after infection were comparable for individual young and aged mice. There were, however, major perturbations in the epitope specificity of the majority of aged mice, in that there was an approximate fivefold reduction in the mean response to NP, with many aged mice completely negative, whereas the mean responses to PA and PB1 were comparable between young and aged mice. Furthermore, our characterization of the T-cell receptor (TCR) repertoire using both Vβ-specific antibodies and DNA spectratype analysis showed a major perturbation in the TCR repertoire of NP-specific T-cells in the aged mice, with a frequent loss of T-cells expressing the dominant Vβ8 T-cell receptor (Deckhut et al. 1993). Thus, we identified an age-associated loss in the ability of individual mice to respond to a normally immunodominant influenza virus epitope. The correlation between low naïve precursor frequency in young mice and reduced thymic output in aged mice was further reinforced by analysis of thymectomized young mice. Thymectomy mimics one effect of aging by eliminating export of new thymic immigrants to the periphery. Our analysis of the repertoire of influenza virus-specific $CD8^+$ T-cells

following infection of mice 7 months postthymectomy (8 months of age) showed that, similar to the response of aged (18-month-old) mice, there was a selective loss of responsiveness to NP. Taken together, these data provide formal evidence for the preferential development of "holes in the repertoire" of aged mice to a normally immunodominant viral epitope having a low naïve precursor frequency.

A key question is whether the loss of a response to NP has implications for the development of protective immunity in aged mice. To test this, we first analyzed the $CD8^+$ T-cell response to NP after primary influenza virus infection (PR8, H1N1) in individual young and aged mice by tetramer staining of peripheral blood, and 30 days later challenged the mice with a heterologous influenza virus that could not be cross-neutralized by antibody (x31, H3N2). Seven days after challenge, a time at which all young primed mice have cleared virus, aged mice were analyzed for viral clearance and epitope specificity. The results showed a remarkable association between the ability to generate a robust response against NP and effective viral clearance. Importantly, individual aged mice in which NP-specific $CD8^+$ T-cells constituted less than 5% of the total $CD8^+$ T cell response failed to clear virus, while those mice with a more robust NP response (5-15% NP-specific CD8 T-cells) had cleared virus on day 7, analogous to the young mice in which the NP-specific $CD8^+$ T-cells constituted between 6-25% of the $CD8^+$ T cell response. These data show that an age-associated loss in responsiveness to specific epitopes is associated with a decline in protective heterosubtypic immunity, providing direct evidence for the contribution of declining $CD8^+$ T-cell repertoire diversity on the loss of immune function with age.

We have yet to experimentally address the second part of our hypothesis; that the response of the elderly to new antigens will be increasingly dominated by fortuitously cross-reactive memory T-cells. However, preliminary data show that memory T-cells isolated from aged naïve mice can respond to specific influenza virus epitopes, supporting the existence of fortuitously cross-reactive memory T-cells that have the potential to respond to de novo influenza virus infection. As expected, the response pattern was distinct in individual animals, reflecting uniqueness in the repertoires of memory cells in each aged mouse. Experiments to further characterize the contribution of fortuitously cross-reactive memory responses in aged mice following de novo influenza virus infection are ongoing.

5 The Impact of Aging on Peripheral Memory T-Cell Pools

In addition to understanding the impact of age on de novo infections, it is also important to understand how age impacts T-cell memory that was originally generated in a young individual. This is a critical question, since many pathogens, such as influenza virus, are first encountered during youth. A key feature of $CD8^+$ T-cell memory to respiratory virus infections is that the efficacy of the recall response declines over the first year in both mice and humans (Ely, Roberts et al. 2007). This declining efficacy is related to the progressive loss of virus-specific $CD8^+$ T_{EM}

cells from the peripheral and systemic memory T-cell pool. Although the absolute number of memory cells in the circulation remains relatively constant over time, there is a gradual conversion of the virus-specific population from a T_{EM} to a T_{CM} phenotype (Hikono et al. 2006, 2007; Hogan, Usherwood et al. 2001; Tripp et al. 1995). The conversion of the population from a T_{EM} to a T_{CM} phenotype, denoted by the re-expression of CD62L over time, appears to represent the specific outgrowth of central memory cells by homeostatic mechanisms rather than a change in phenotype in individual cells (Marzo et al. 2005). There is some evidence that the selective outgrowth of central memory T-cells may occur in the bone marrow (Becker et al. 2005). The expression of the activation-associated glycoform of CD43 also changes with similar kinetics, progressing from a mixed $CD43^{high}$ and $CD43^{low}$ phenotype to an exclusively $CD43^{low}$ phenotype. Thus, it would appear that memory T-cells ultimately progress toward a more quiescent $CD62L^{high}/CD43^{low}$ phenotype.

Despite a negligible impact on the total number of virus-specific memory $CD8^+$ cells, phenotypic conversion of the memory T-cell population can have a dramatic impact on the kinetics and efficacy of a recall response. This conversion results in the progressive loss of T_{EM} cells from peripheral sites such as the lung (phase 1 responders) and a reduced capacity to mediate the rapid recruitment of memory T-cells early during the recall response (phase 2 responders). The decline in these early phases of the recall response results in a diminished capacity to clear a secondary virus infection in the lung. In mouse models, the loss of peripheral memory takes about 8 months to a year. A similar loss of peripheral memory occurs in humans, although the kinetics are less clear. In light of the relatively rapid loss of peripheral memory, it should be emphasized that this process is not a direct effect of aging and occurs in both young and aged animals alike.

While the loss of peripheral T_{EM} cells with age results in a decline in mucosal immunity, it is less clear whether age also impacts the ability of systemic T_{CM} cells to mediate recall responses. We have addressed this question by comparing the capacity of recent versus long-term memory $CD8^+$ T-cells to proliferate in response to Sendai virus infection in vivo using a dual transfer approach (Ely et al. 2003; Roberts and Woodland 2004). In these experiments, memory cells were isolated from the spleens of donor mice that had recovered from a prior Sendai virus infection (either 1-month or 12-month postinfection) and cotransferred into Sendai virus infected recipient mice. These two populations were then compared for their capacity to generate recall responses in the lungs of the same host. These studies demonstrated that although both donor populations proliferated strongly, the 12-month donor memory cells generated much stronger responses than the 1-month donor memory cells (on a per cell basis). Importantly, the dominance of 12-month memory $CD8^+$ T-cells could not be attributed to better $CD4^+$ T-cell help since both the 1-month and 12-month recall responses occurred within the same animal in the presence of the host Sendai virus-specific $CD4^+$ T-cell response. Thus, these data indicated that memory T-cell populations actually improve with age in terms of their capacity to mediate proliferative recall responses in the lung.

One possible explanation for the increased capacity of aged memory T-cells to mediate recall responses is the progressive accumulation of T_{CM} cells, which may have an enhanced capacity to proliferate in response to antigen However, using the

dual adoptive transfer approach, we showed that the improved capacity of long-term memory cells to mediate proliferative responses occurred in both the $CD62L^{lo}$ (T_{EM}) and $CD62L^{hi}$ (T_{CM}) subsets. This led to the speculation that other T-cell subpopulations could explain the differences in the response of recent versus aged memory. In this regard, we have demonstrated that a subpopulation of memory T-cells express the killer cell lectin-like receptor G1 (KLRG1; Hikono et al. 2007). KLRG1 is a marker of senescent cells that exhibit poor in vivo proliferative capacity, but do not express programmed death-1 (PD-1), a marker of T-cell exhaustion (Barber et al. 2006; Voehringer et al. 2001; Zajac et al. 1998). $KLRG1^{+}/PD\text{-}1^{-}$ cells can represent up to 40% of a newly established memory T-cell pool and transfer studies confirmed that these cells have only a limited capacity to mediate recall responses (Hikono et al. 2007; Voehringer et al. 2001). Thus, newly generated memory contains a substantial fraction of these poorly responsive cells. Over the long term, this population declines, leading to an 'enrichment' of the remaining memory T-cell pool due to increasing frequencies of highly responsive T-cells. Consistent with this, long-term memory cells from aged mice were more responsive than recently generated memory cells on a per-cell basis at mediating recall responses. In other words, the memory T-cell pool actually improved in its capacity to mediate recall responses in the lung with increasing age due to the gradual loss of poorly proliferative $KLRG1^{+}$ cells. Note that in these experiments, animals exhibiting T-cell expansions were purposefully excluded to avoid complications (*See* below).

6 Dysregulation of T-Cell Memory with Age

As discussed above, the capacity of memory T-cell pools to mediate recall responses is clearly differentially affected by age. Whereas the overall efficacy of the response is reduced due to the decline in peripheral memory, there is also a corresponding increase in the capacity of systemic memory to mediate proliferative responses on a per cell basis. An additional complication is that the immune repertoire is further dysregulated by the appearance of $CD8^{+}$ T-cell clonal expansions (TCE). TCE are nonmalignant monoclonal populations of $CD8^{+}$ T-cells that appear with increasing frequency as individuals age (Callahan et al. 1993; Clambey et al. 2005; Effros et al. 2003; Hingorani et al. 1993; Messaoudi et al. 2004; Posnett et al. 1994, 2003). The sizes of these expansions are variable, but they can sometimes represent up to 90% of the entire peripheral T-cell repertoire. TCE in humans strongly correlate with seropositive responses to chronic virus infections, such as cytomegalovirus, suggesting that persistent antigenic stimulation drives these expansions (Khan et al. 2002; Koch et al. 2006). However, TCE are also observed in humans and mice that lack any obvious persistent infection (Clambey et al. 2005). These data suggest that TCE may fall into two distinct categories that are either dependent or independent of chronic antigenic stimulation (Messaoudi et al. 2006).

The requirement for antigen in the generation and maintenance of TCE in the absence of a persistent infection is enigmatic. We have analyzed this question using mouse models of acute Sendai and influenza virus infections. Intranasal infection

of mice with these viruses elicits a strong $CD8^+$ T-cell response that clears virus from the lungs within 10 days of infection. Antigen-specific memory $CD8^+$ T-cells specific for immunodominant epitopes persist for life in the animals and typically represent less than 1% of the total $CD8^+$ T-cell pool. However, 18 months postinfection, $CD8^+$ TCE specific for the different immunodominant Sendai and influenza virus epitopes were found to emerge with increasing frequency, in some cases representing greater than 90% of the $CD8^+$ T-cell pool. Spectratype and T-cell receptor Vβ analysis confirmed that these TCE were monoclonal in nature (Ely et al. 2007). In general, these TCE expressed phenotypes typical of conventional memory cells and were present in both the T_{EM} and T_{CM} subsets. TCE were also functional in terms of their capacity to produce cytokines and to proliferate in vitro in response to cognate antigen or homeostatic cytokines (Li et al. 2005; Zhang et al. 2002). These observations suggest that TCE can arise from the entire memory T-cell pool, however we cannot formally rule out that the TCE identified arose from a particular subset and subsequently changed their phenotype. Importantly, the appearance of these cells cannot be readily explained by persistent antigen since infectious virus is cleared within 10 days and there is no evidence for antigen persistence longer than a few weeks (Jelley-Gibbs et al. 2005; Zammit et al. 2006; Hou et al. 1992; Usherwood et al. 1999). While the antigen-specific TCE that develop in mice that have recovered from either Sendai or influenza virus infections are clearly antigen-specific, it is tempting to speculate that expansions of unknown specificity also arise from the memory T-cell pools that are present even in specific pathogen free mice. Taken together, these data were the first to demonstrate that at least some TCE can develop from the conventional memory T-cell pool elicited by an acute (as opposed to persistent) pathogen infection.

The mechanisms by which TCE develop are unclear. Homeostatic proliferation appears to play an important role, although the rate of proliferation of a given TCE is not overtly different from that of the general memory T-cell pool. Consistent with this idea, TCE typically express receptors for cytokines that are involved in $CD8^+$ T-cell homeostasis, namely CD122 (IL2Rβ/IL15Rβ) and CD127 (IL7Rα). Furthermore, antigen is not required for the maintenance of antigen-specific TCE since they continue to proliferate and maintain their numbers following transfer into mice in which the cognate antigen is absent (Clambey et al. 2005; Ely et al. 2007). A more detailed analysis of the memory T-cell pool indicates that large TCE may simply be the most prominent feature of a general breakdown or dysregulation of the memory T-cell pool. The impression is that TCE are constantly emerging and expanding to varying degrees, resulting in a progressive decline in the overall diversity of the memory T-cell pool. For example, using memory T-cell pools in young mice as a standard, approximately 75% of aged mice (greater than 18 months old) expressed perturbations that were two standard deviations outside the normal range for young mice and were therefore considered potential TCE. Thus, one possible explanation for the appearance of TCE is that they reflect a gradual dysregulation of the normal process of homeostatic proliferation that operates to maintain memory T-cell numbers. In other words, TCE may be a natural outcome of the long-term homeostatic proliferation of the memory T-cell pool. This would become evident on

a population level if individual clones proliferated at slightly different rates, causing the overall memory T-cell pool to become progressively less diverse over time and leading to the emergence of TCE. While an attractive idea, it should be noted that some TCE appear to expand and then subsequently regress within the same individual (Clambey et al. 2005). This means either that different TCE are regulated by distinct mechanisms or that there are universal control mechanisms that can come into play.

The extent to which TCE are able to impact immune responses is not well understood. TCE generated by chronic viral infections, and presumably by persistent antigenic stimulation, appear to be functionally impaired as they fail to produce effector cytokines upon restimulation in vitro (Clambey et al. 2005; Khan et al. 2002; Koch et al. 2006; Messaoudi et al. 2004; Posnett et al. 2003). In addition, TCE that expressed the same Vβ as a dominant portion of the normal response to HSV-1 were shown to have a Vβ-specific negative impact on the subsequent de novo response to HSV-1 (Messaoudi et al. 2004). In the case of antigen-specific TCE, their presence will generally be detrimental due to the fact that they severely compromise the overall size, and therefore the diversity, of the rest of the T-cell pool. This will have the effect of reducing the number of memory cells specific for other antigens or pathogens. However, this hypothesis has yet to be proven. It may also be the case that their high frequencies could actually benefit a response to the pathogen for which they are specific. This may be relevant for a pathogen like influenza in which an individual may encounter the same T-cell epitope during multiple infections over the course of many years.

7 Implications for Vaccination

It is well-established that the elderly are difficult to vaccinate, and efforts to improve vaccination efficacy are a high priority. Our data suggest that declining repertoire diversity and the potential to develop "holes in the repertoire" can have a profound effect on strategies to boost effectiveness of vaccination. For example, the use of epitope-based vaccine strategies would be counter-intuitive for aged individuals since naïve T-cell precursors capable of recognizing a particular epitope may be missing. In addition, the use of potent adjuvants in the elderly may be both ineffective and dangerous, because a lack of naïve T-cell precursors could allow for the boosting of unwanted, pathological cross-reactive responses (Selin et al. 2006). Instead, a more proactive approach earlier in life may result in efficacious cellular immunity among elderly individuals. For example, because memory generated in young individuals persists with time (Haynes et al. 2003; Kapasi et al. 2002; Roberts et al. 2005), one rational approach is to enhance vaccination efficacy of young adults by focusing on strategies that will generate robust and diverse T-cell responses that will persist into old age. This enhancement will involve the administration of vaccines that elicit large numbers of pathogen-specific memory T-cells, while limiting the number of poorly responsive ($KLRG1^+$) cells that could dampen protective immunity during a recall response. In addition, efforts to prolong thymic

output and/or the lifespan of naïve T-cells, or to reconstitute naïve T-cells in the elderly, warrant further investigation (Beverley and Grubeck-Loebenstein 2000; Goronzy et al. 2007; R. D. Kovaiou et al. 2007; McElhaney 2005; Nikolich-Zugich 2005; van den Brink et al. 2004). The prolonged availability of naïve T-cell precursors could allow for continued boosting of the response through vaccination, in turn renewing the peripheral T_{EM} population capable of mounting immediate responses at the site of virus infection.

Clearly, the last several years have seen considerable progress in the elucidation of the dynamics of T-cell memory during aging, and the underlying defects that hamper the establishment of quality T-cell memory in aged individuals. As we move forward, the identification of the mechanisms that govern these processes will be of crucial importance. A thorough understanding of these mechanisms will be necessary for the development of rational vaccination strategies for the elderly.

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HIV Infection as a Model of Accelerated Immunosenescence

Victor Appay and Delphine Sauce

Contents

Abstract: Since its discovery in 1983, HIV-1 has become the most extensively studied pathogen in history. Massive $CD4^+$ T-cell depletion and sustained immune activation and inflammation are hallmarks of HIV-1 infection. However, the precise pathway to the onset of immunodeficiency that develops during HIV-1 infection has not been resolved yet. In recent years, an intriguing parallel between HIV-1 infection and ageing has emerged: HIV-1 infected individuals present immunological alterations that are remarkably similar to those accumulated with age by HIV-1 uninfected elderly. These alterations, e.g., loss of regenerative capacity and accumulation of ageing T-cells, are suggestive of a process of immunosenescence,

V. Appay (✉) · D. Sauce
Cellular Immunology laboratory
INSERM U543, Avenir Group
Hopital Pitie-Salpetriere
Université Pierre et Marie Curie-Paris6
91 Bd de l'Hopital, 75013 Paris, France
Tel.: 00 33 1 40 77 81 83
Fax: 00 33 1 40 77 97 34
E-mail: victor.appay@chups.jussieu.fr

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which may result from persistent HIV-1 replication and systemic immune activation. Furthermore, the comparison between HIV-1 infection and human ageing may go beyond the sole onset of immunosenescence, and extends to the deterioration of a number of physiological functions related to inflammation and systemic ageing. In the present chapter, we provide to the readers the different pieces of the HIV pathogenesis puzzle, from the virus itself to the development of therapeutic strategies, and discuss how they fit together into a model of accelerated immunosenescence and systemic ageing in HIV infection.

Keywords: HIV pathogenesis • $CD4^+$ T-cells • Immune activation • Regenerative capacity • Exhaustion

1 Introduction

In 1981, physicians in the United States reported an unusual outbreak of infections in the homosexual community: previously healthy homosexual men were dying from infections that should normally be resolved with no problem [1, 2]. Within a year, this condition, characterized by markedly reduced circulating $CD4^+$ T-cell counts, was referred to as acquired immunodeficiency syndrome or AIDS. The pathogen responsible for AIDS was identified as a T-lymphotropic retrovirus for the first time in 1983 by a French group [3], followed by groups in the US [4, 5]. Formerly known as lymphadenopathy-associated virus (LAV), human T-lymphotropic virus Type III (HTLV III) or AIDS related virus (ARV), the virus was named human immunodeficiency virus or HIV. Retrospectively, the first recorded case of HIV-1 infection was reported in a blood sample taken in 1959 in former Zaire [6].

Over the past 50 years, from isolated case reports, the scale of the HIV epidemic has become a global pandemic, and HIV the most extensively studied and notorious pathogen in history. The World Health Organisation (WHO) and UNAIDS estimate that close to 40 million people were living with HIV by the end of the 2006, with Africa accounting for the great majority of these cases. For instance, in Bostwana, the prevalence of infection is estimated to be about one third of the adult population, and in Namibia, life expectancy has dropped from 61 years in 1991 to 45.5 years in 1996. In addition to its disastrous impact on the African population, HIV is very likely to have profound consequences on the economies and therefore on the future of these developing countries.

Most surprisingly, such devastation is caused by a rather small virus, consisting of only 9 genes. Scientists had originally anticipated the rapid development of effective vaccines and cures against this virus. However, the solution to the HIV pandemic is still to come. In reality, the precise reasons for the onset of immunodeficiency that almost inevitably develops during HIV infection, or the exact process through which HIV leads to AIDS, have not been resolved yet. The relationship between HIV and its host has emerged as extremely complex: immunologic, genetic, viral and environmental factors, can potentially contribute to the rate of HIV disease progression. However, 25 years of intense research have not been futile: pieces of

the puzzle are starting to come together and the whole picture of HIV pathogenesis is being unraveled little by little. The infection of $CD4^+$ T-cells, key players of the immune system, by HIV and its capacity to mutate rapidly in order to escape its host immunity are central features of the virus efficacy to persist and cause severe damage. However, in recent years, 2 further points or observations have emerged as being potentially fundamental in HIV pathogenesis: the role of immune activation and inflammation, and an intriguing parallel between HIV infection and ageing.

In the present chapter, our aim is to present to the readers the different pieces of the HIV pathogenesis puzzle and discuss how they fit together into a model of accelerated immunosenescence and systemic ageing in HIV infection.

2 Important Properties of HIV-1

2.1 Infection with HIV-1

HIV belongs to the lentivirus subgroup of retroviruses. Two HIV viruses have been described: HIV-1 and HIV-2. Infection with HIV-1 is highly prevalent worldwide and is responsible for the HIV pandemic. HIV-2 infection is observed only in some countries of West Africa and usually results in a mild disease course. HIV is an enveloped RNA virus whose structural components can be broadly divided into the viral envelope, matrix and core, viral RNA and enzymes. Its genome consists of only 9 genes divided into 2 structural genes (*gag* and *env*) encoding the capsid and envelope proteins, one gene (*pol*) encoding enzymes necessary for the HIV replicative cycle (i.e. reverse transcriptase, integrase, and protease), and a series of accessory genes (*nef, rev, tat, vpu, vif* and *vpr*). Moreover the long terminal repeat (LTR) found at each end of the virus is responsible for encoding binding sites for the initiation of virus transcription, as well as for utilizing cellular transcription factors after the virus has been integrated into its host genome [7].

Infection with HIV occurs primarily by sexual contact (heterosexual as well as homosexual), transfer of infected blood or transmission from mother to child. In order to enter cells and initiate its replicative cycle, HIV requires that its target expresses two separate molecules: CD4, which acts as the primary HIV receptor; and a second co-receptor, generally either CCR5 or CXCR4. Several cell populations present in the genital tract express the requisite receptors for productive HIV-1 infection, including CD4 lymphocytes, Langerhans cells and macrophages. The main cell target during established HIV-1 infection is the activated $CD4^+$ T-lymphocyte [8]. After productive infection by HIV-1, there are still several possible fates for an infected host cell. Most HIV-infected, activated cells will die quickly ($t_{1/2}$ 1 day), through direct cytopathic effects of the virus, apoptosis or killed by the host antiviral immune response [9, 10]. However, a fraction of these T-cells, carrying integrated proviral DNA, revert back to a resting state. These $CD4^+$ lymphocytes constitute a stable pool of long-lived memory T-cells, which are not transcribing viral genes and so are not susceptible to killing by host HIV-specific cellular effectors. These

latently infected cell pool constitutes a reservoir for the virus and the integrated HIV-1 genome will actively replicate upon cell activation [8]. HIV-1 can spread to the whole lymphatic and blood system, including lymphoid organs like lymph nodes or the thymus.

2.2 *The Course of HIV-1 Infection*

The clinical course of HIV-1 infection can be divided into three distinct stages: an early, acute stage; a middle, chronic stage; and a late, immunodeficiency stage (AIDS) (see Fig. 1a). During the latter stage, opportunistic illnesses develop that are characteristic of AIDS, and if untreated this stage will progress to death. Acute HIV-1 infection is characterized by extensive viral replication and dissemination, prior to the induction of host immune responses, resulting in a transient CD4+ lymphocytopaenia. The symptoms and high viremia associated with primary infection generally decline

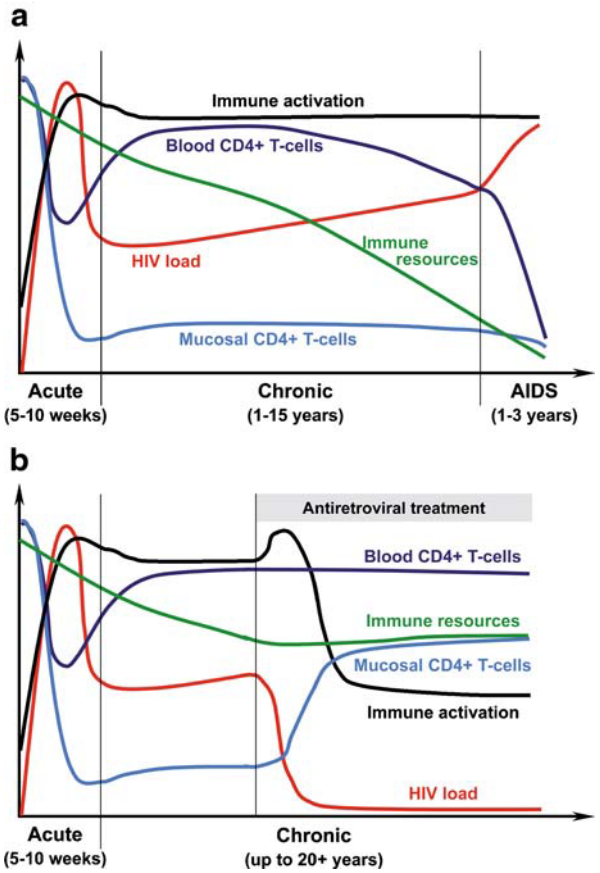

Fig. 1 (a) The course of natural HIV-1 infection (b) The course of HIV-1 infection with antiretroviral therapy

within a few weeks, as the host cellular immune response reduces plasma viremia. With the resolution of the clinical symptoms associated with primary infection, CD4+ counts generally return to more normal levels; the initial viremia is followed by the establishment of a viral set point, an equilibrium between virus and host [11], defining the chronic stage of HIV-1 infection, that can last for up to several years. This phase of HIV-1 infection is associated with no (or minimal) clinical symptoms, although CD4+ T-cell counts fall gradually [12]. The late stage of HIV infection is AIDS, manifested by a decline in the number of CD4+ cells to below 200/mm^3 and a raise in plasma viremia, eventually resulting in immunologic collapse and the incidence of opportunistic infections. The time taken to progress from clinical latency to AIDS, in the absence of antiretroviral therapy, has a median of 10 to 11 years in developed countries, and 3 to 4 years in developing countries. The higher the viral set point, the faster the individual is to progress to symptomatic AIDS. The 2 most characteristic manifestations of AIDS are Pneumocystis pneumonia and Kaposi's sarcoma. However, many other opportunistic infections occur with some frequency. These include viral infections such as disseminated herpes simplex, herpes zoster, and cytomegalovirus infections and progressive multifocal leucoencephalopathy; fungal infections such as thrush, cryptococcal meningitis, and disseminated histoplasmosis; protozoal infections such as toxoplasmosis and cryptosporidiosis; and bacterial infections such as disseminated Mycobacterium avium-intracellulare and Mycobacterium tuberculosis infections. Many AIDS patients have also severe neurologic problems, e.g., dementia and neuropathy. Unless the underlying immunodeficiency can be addressed by the use of antiretroviral drugs [13], subjects who have reached the stage of AIDS will generally die within one to two years [12].

2.3 Mechanisms of Persistence

HIV is one of the most successful viruses to subvert and manipulate the host immune system, achieving life long chronic infection. Thorough studies have permitted to reach a good understanding of the reasons why the immune system fails to eradicate completely the virus after primary infection. HIV-1 possesses a range of mechanisms to escape its host immunity and to establish persistence.

The replication process of HIV-1 using the error prone enzyme reverse transcriptase can generate around 1 point mutation per 10^4 nucleotides, meaning that each progeny virus will potentially contain at least one base site mutation. It is estimated that an infected person can produce up to 10 billions new virions each day. This leads to an amazing capacity to produce new mutants during the entire course of an HIV infection [14]. HIV-1 is therefore able to mutate under selection pressure from the immune system, resulting in the rapid emergence of variants that can elude both antibody and T-cell recognition [15, 16]. For instance, mutations appear in the sequence of viral epitopes normally recognized by CD8+ T-cells, affecting the binding of the peptide to the MHC Class I molecule, and resulting in loss of CD8+ T-cell detection and stimulation. In some case, the development of escape mutants correlates directly with disease progression [17, 18].

HIV-1 expresses also factors known to enhance pathogenicity. The most studied of these factors is Nef. Nef is able to downregulate the surface expression of surface MHC Class I [19, 20] and to upregulate FasL [21], providing a possible role in evasion of the immune response. Moreover, Nef induces a 5 to 10 fold increase in the endocytosis of the CD4 molecule, followed by transport to the lysosomes [22]. This may facilitate the generation of new virions, as intracellular CD4 interferes with gp120 incorporation into the virus [23, 24]. Another HIV protein, Vpu, has been shown to induce degradation of CD4 molecules by targeting CD4 to the proteosome [22]. Vpu also prevents the cell surface expression MHC Class I molecules, by disrupting its processing [22].

Finally, as seen earlier, HIV-1 establishes latent reservoirs in resting memory cells that confers on the virus the capacity to remain hidden from immune surveillance and to pursue low levels of replication throughout the lifetime of the infected person [25]. Although these mechanisms are exclusive to HIV-1, the ability to persist in its host and to establish chronic infection is not unique to HIV-1, as a range of other viruses (e.g., Herpes and Hepatitis viruses) have also developed means to escape their host immunity and establish successful persistence.

2.4 HIV-1 Mediated Depletion of CD4⁺ T-cells

What then makes HIV-1 different from other persisting viruses which do not lead to a general process of immunodeficiency? HIV is unique in that it targets the CD4+ T-cell pool (as well as, but to a lesser extent, macrophages and dendritic cells), which holds an essential role in immunity. The infection and depletion of CD4+ T-cells represents the most fundamental event in the pathogenesis of HIV-1 infection. Acute HIV-1 infection is characterized by a transient and modest decrease of CD4+ T-cell count as observed in the peripheral blood. However, this is not representative of the total body CD4+ T-cell count since the majority of CD4+ T-cells resides actually not in peripheral blood, but in lymphoid tissues like the lymph nodes, and in particular, the mucosal lymphoid tissues like the gastrointestinal tract. Mucosal CD4+ T-cells consist predominantly of memory CD4+ T-cells which express the HIV co-receptor CCR5 and present relatively activated status [26–28]. They are therefore ideal targets for the virus. Importantly, studies performed in primates infected with SIV (the simian equivalent of HIV) as well as in HIV-1 infected humans revealed that massive CD4+ T-cell depletion takes place in mucosal tissues during acute infection [28–30], due to direct target cell infection [31, 32] and apoptosis [33]. As a consequence, the gut associated mucosal tissue becomes the most important site of active viral replication and T-cell depletion during acute infection. It is thought that HIV infected patients can lose the majority of their CD4+ T-cell pool (60 to 80%) within the first few weeks post infection. This massive depletion of CD4+ T-cells, which is maintained throughout all stages of HIV infection [28], is obviously not left without consequences, as discussed later.

HIV-1 infected individuals are also characterized by a gradual decline of peripheral blood CD4+ T-cell counts during chronic infection, which, although it does not reflect the massive depletion of mucosal CD4+ T-cells, is critical in HIV pathogenesis. It is directly associated with HIV disease progression: low circulating CD4+ T-cells count coincides best with the onset of AIDS, as minimum levels of CD4+ T-cells are required to maintain immune integrity. The frequency of infected circulating CD4+ T-cells during chronic infection is too low (0.01 to 1%) to account solely for this general decline of peripheral blood CD4+ T-cells [34–36]. Activation induced apoptosis (discussed soon after) is actually considered as a major cause of peripheral CD4+ T-cell depletion in HIV infected patients. Moreover, CD4+ T-cell depletion takes place in a context of impaired or reduced T-cell renewal, so that a significant proportion of depleted CD4+ T-cells will eventually not be replaced, hence this progressive decline.

3 Immune Activation and Inflammation in HIV-1 Infection

3.1 The Paradoxical Immune Activation

Progressive CD4+ T-cell depletion is the hallmark of HIV-1 infection. However, another phenomenon has become apparent: the association between HIV infection and chronic immune activation and inflammation. HIV infected individuals display elevated markers of activation and apoptosis of CD8+ and CD4+ T-cells [37–40], as well as B-cells, NK-cells and monocytes. High levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6) and interleukin 1 beta (IL-1β) in both plasma and lymph nodes, are also observed from the early stages of HIV-1 infection [41–45]. The secretion of chemokines like MIP-1α, MIP-1β and RANTES is increased in these patients [46, 47]. Immune activation, which usually reflects the mounting of antiviral immunity, may be seen as a normal and positive observation in the case of an infection with any pathogen including HIV. However, in the 90s, Giorgi and colleagues reported a rather counter intuitive observation: T-cell activation levels, as measured with the expression of the activation marker CD38 on CD8+ T-cells, were predictive of an adverse prognosis for the infected patients [48–50]. Several investigators have then confirmed that there is indeed a direct correlation between HIV-1 disease progression and CD8+ T-cell activation levels [51–53].

Further evidence of the paradoxical role of immune activation in HIV infection was brought by studies of SIV infected primates. Rhesus macaques which, like HIV infected humans, suffer progressive CD4+ T-cell depletion and progression to AIDS upon SIV infection, are characterized by strong T-cell activation. In contrast, SIV infected Sooty mangabeys and African green monkeys, which do not develop any immunodeficiency, exhibit minimal T-cell activation despite evident viral replication [54]. Another interesting observation comes from the study of HIV-2 infected individuals: HIV-2 infection leads to a mild or slow disease progression, and most

HIV-2 infected patients will die from HIV-2 unrelated causes; interestingly these patients display significantly less immune activation compared to HIV-1 infected individuals [55]. The adverse effect of immune activation in HIV pathogenesis may also account for the observations linking more rapid disease progression in Kenyan prostitutes and frequent intercurrent infections and related immune activation [56], or for the accelerated SIV-induced disease progression reported in SIV-infected macaques which were subjected to repeated SIV-independent immune stimulus to mimic chronic activation [57].

3.2 The Causes of Immune Activation and Inflammation in HIV-1 Infection

During HIV-1 infection, immune activation and inflammation involve several mechanisms, which are both directly or indirectly related to viral replication. The common cause of T-cell activation during an infection is antigenic stimulation by the virus, which is the foundation of the adaptive immune response. During primary infection, HIV-1 induces strong T-cell responses, in particular CD8+ T-cells, which can persist during the chronic infection phase due to the continuous replication of the virus: up to 20% of circulating CD8+ T-cells can be HIV specific in untreated chronically infected patients [58, 59]. HIV specific CD4+ T-cell responses are usually present at a lower magnitude (i.e., up to 3% of circulating CD4+ T-cells) [58], which may be related to their preferential depletion by the virus [35].

Nonetheless, the extent of activation during the course of HIV-1 infection is such that stimulation with HIV antigens solely cannot account for the complete phenomenon of immune activation observed. Although the physiological impact is not known yet, in vitro studies suggest that HIV gene products can induce directly the activation of lymphocytes and macrophages, and the production of proinflammatory cytokines and chemokines. The envelop protein gp120 may be able to activate cells or to enhance their responsiveness to activation, even in absence of direct infection, through binding to CD4 or coreceptors [60–62]. The accessory protein Nef is also able to lead to lymphocyte activation either directly [63, 64] or through the infection of macrophages [65].

HIV-1 causes also immune activation and inflammation through indirect means. Antigenic stimulation during HIV-1 infection may be induced by other viruses, like CMV and EBV. CMV reactivation appears to occur recurrently in healthy donors as evidenced by the presence of a large population of CD69+ CMV specific cells indicative of recent *in vivo* activation [66]. During HIV-1 infection, the depletion of CD4+ T-cells may result in suboptimal immune control of these persistent viruses and thus permits their reactivation and replication. In addition, inflammatory conditions occurring during HIV infection (e.g., release of proinflammatory cytokines) may also participate in the reactivation of latent forms of CMV and EBV. Recent studies have shown significant activation of EBV and CMV specific CD8+ T-cells during HIV-1 acute infection [67, 68]. Hence, sustained antigen mediated immune

activation occurs in HIV-1 infected patients, which is due to HIV-1, but also to other viruses (and may not be only restricted to CMV and EBV).

Recently, Douek and Brenchley have brought to light another potential mechanism that could be central in HIV pathogenesis and involves the activation of innate immune system [69]. The massive depletion of CD4+ T-cells (and possibly macrophages and dendritic cells) by HIV-1 in mucosal lymphoid tissues can result in disrupting the different immune components that constitute the mucosal barrier. This barrier usually prevents the translocation of the flora that inhabits the intestinal tract and restricts these pathogens to the lamina propia and the mesenteric lymph nodes. Compromising its integrity may therefore results in microbial translocation from the gut to the systemic immune system [70]. Interestingly, HIV-1 infection is associated with a significant increase of plasma LPS levels, an indicator of microbial translocation. Plasma LPS is directly correlated with measures of immune activation [69]. Translocation of bacterial products is highly likely to result in a profound activation of the innate immune response: for instance lipopolysaccharide (LPS), flagellin, and CpG DNA, which are toll like receptor (TLR) ligands, are known to directly stimulate peripheral macrophages and dendritic cells to produce a range of proinflammatory cytokines (e.g., TNFα, IL-6 and IL-1β). The eventual outcome is bystander activation and differentiation of lymphocytes and monocytes, and the establishment of a proinflammatory state.

4 The Consequences of Immune Activation and Inflammation in HIV-1 Infection

The initiation of this state of immune activation and inflammation and its long term establishment due to persistence of the virus have extensive and detrimental effects on the immune system and human health.

4.1 The Vicious Cycle of Immune Activation and HIV-1 Spreading

A direct consequence of T-cell activation is the increase of intracellular nuclear factor kappa B (NF-κB) levels, which enhances the transcription of integrated virus, and therefore the production of new virions that will infect new targets [71]. A vicious cycle is therefore established during which HIV-1 replication promotes immune activation, and immune activation promotes HIV-1 replication. Released proinflammatory cytokines participate also to this refueling cascade: the synergic action of IL-1β, TNFα and IL-6 can lead to T-cell activation [72]; IL-1β and TNFα may also decrease transepithelial resistance in mucosal tissues, therefore promoting microbial translation [73].

Immune activation implies enhanced T-cell turnover, differentiation from naïve to antigen experienced cells, and apoptosis. A large number of T-cells end up dying upon activation, independently from HIV infection. However, dynamics of activation, expansion and apoptosis seem to differ between CD4+ and CD8+ T-cells [74–76]. CD8+ T-cells experience extensive expansion upon activation and can establish a stable pool of resting memory cells. In contrast, the capacity of CD4+ T-cells to expand and survive seems to be lower, so that the vast majority of activated CD4+ T-cells apoptose rapidly, hence a further burden with regard to the renewal of the CD4+ T-cell pool. Overall, the immune system of HIV-1 infected individuals faces major difficulties: it has to cope with a massive cellular destruction, in particular CD4+ T-cells (through apoptosis or direct infection), and to contain HIV-1 replication, as well as associated pathogens. Dealing with such overwhelming and enduring challenge has a cost.

4.2 Immunosenescence and HIV-1 Infection

4.2.1 The Limited Regenerative Capacity of the Immune System

Considering the multiplicity of pathogens the immune system must face throughout a life time, its plasticity and efficacy are prodigious. The complete reconstitution of the pool of immune cells from a small number of precursors in the context of leukemia/lymphoma treatment (i.e., infusion of hematopoietic stem cells after chemotherapy and total body irradiation) has highlighted the fantastic capacity of the immune system to regenerate itself. Nonetheless, this capacity may have boundaries. Accumulating evidence suggests that the so-called Hayflick limit (i.e., the irreversible state of growth arrest indicative of replicative senescence, initially observed with cultured human fibroblasts) applies to cells of the immune system [77], so that their replicative lifespan in vivo is limited. The occurrence of replicative senescence is primarily related to the number of cell divisions. A commonly used marker of replicative history is the length of the telomeres (repeated hexameric DNA sequences found at the ends of the chromosomes), that is reduced with each cell division. Important telomere shortening can result in chromosome instability and eventually in growth arrest and/or apoptosis of the cells. During primary viral infection, upregulation of telomerase (the enzyme involved in the maintenance of telomere length) occurs in order that activated virus specific T-cells maintain telomere length despite the considerable clonal expansion which takes place at that moment [78, 79]. However, such capacity to upregulate telomerase seems to decrease after repeated stimulation [80], so that memory T-cells specific for persisting viruses will eventually present shorter telomere length, as exemplified in EBV infection [81, 82], and reach stages of replicative senescence. The immune system deals with this irreversible exhaustion of T-cells by continuously providing new cells.

However, the thymus (the organ on which depends the generation of naïve T-cells and the maintenance of TCR diversity [83]), is known to involve with time,

so that it has almost completely disappeared by the age of 60 in humans [84, 85]. Evidence show that the age related changes in the thymus are quantitative rather than qualitative [86]: the rate of naive T-cell output from the thymus dramatically declines with age [85, 87, 88]. Limitation of T-cell regenerative capacity may also occur further upstream in the development of lymphocytes. All the cells which constitute our immune system originate from bone marrow derived hematopoietic stem cells which differentiate and commit themselves to one specific cellular lineage (e.g., myeloid or lymphoid) to generate new granulocytes or naïve lymphocytes continuously. However, emerging data suggest that deregulation of hematopoiesis can occur overtime (i.e., with age). Progenitor cells in elderly individuals present shorter telomeres than in cord blood of newborns [89]. Poor results of bone-marrow transplantation in elderly individuals [90] suggest also that the aged bone-marrow microenvironment has a significantly reduced ability to support hematopoietic regeneration. Moreover, granulocytes and/or naïve T-cells show a shortening in telomere length associated with age [91], or after bone-marrow transplantation [92], suggesting that this applies also to hematopoietic stem cells. Although it is unclear whether this phenomenon has a real consequence on the immune function in ageing, these data support the idea that the regenerative capacity of the progenitor pool may not be unlimited and could reach exhaustion overtime. The overall deterioration of the immune system with time is referred to as immunosenescence. A number of alterations, which characterize HIV infected individuals, seems actually to be related to immunosenescence, as initially supported by Effros and colleagues [93, 94]. This is the likely consequence of immune activation, manifested at two distinct levels.

4.2.2 Senescence/Exhaustion of T-cell Responses

Levels and/or recurrency of cellular activation is a major factor, driving proliferation and T-cell differentiation resulting in the generation of antigen experienced cells, that eventually lack expression of CD28, and show increasing expression of CD57 [67, 95]. These subpopulations tend to lose the capacity to produce IL-2 and present a decline of their proliferative capacity, associated with a shortening of telomere lengths, so that highly differentiated cells ($CD28^-$/CD57+) have been considered as approaching end-stage senescent cells [67, 96]. HIV specific CD8+ T-cell populations play a major role in holding back HIV spreading. These populations are heterogeneous, and consist of cells which can vary in their anti-viral efficacy. For instance, long term non progression may be established through the action of certain populations of HIV specific CD8+ T-cells that display polyfunctional characteristics [97] and/or proliferative capacity [98], and are able to maintain low viral load in infected patients. Avidity of antigen recognition by antigen-specific CD8+ T-cells correlates also with the efficiency of antigen recognition as shown in several antigenic systems [99, 100], and can be one of the main parameters that determines the efficacy of antiviral immunity [101]. However, due to persistent viral replication and repeated stimulation, HIV specific CD8+ T-cells may be gradually driven towards

an irreversible exhaustion of their replicative capacities to become worn-out cells, even resulting in the loss of important anti-HIV T-cell cell subpopulations [101]. Due to their sensitivity for the antigen, high avidity T-cells may be particular sensitive to such stimulation driven depletion. The exhaustion and loss of these "high quality" T-cells may play a significant role in the onset of HIV disease progression, despite other HIV specific CD8+ T-cells, still functionally active but less effective (of lower avidity/efficacy), remain present in the patients [101]. It is important to make the distinction between this irreversible loss of cells and the recently reported exhaustion of HIV specific CD8+ T-cells, based on the expression of PD-1 [102, 103]. The latter may actually be more regarded as a reversible decrease of T-cell functions, as previously described [104, 105] and its upregulation could even reflect T-cell activation due to high viral load, rather than exhaustion [106].

4.2.3 Global Exhaustion of Regenerative Capacity

It is important to appreciate that activation driven immune exhaustion in HIV infection may go far beyond the simple loss of virus specific CD8+ T-cells but extend to a global decline of the immune resources. Although data are still emerging and reasons unclear, HIV infection appears to result in a deregulation of hematopoiesis (lower numbers of progenitor cells and decline in their ability to generate new cells) [107–109]. The capacity of the thymus to produce new cells is also significantly reduced in HIV infected individuals [86]. Several reasons may account for this decline of thymic output: the direct infection of the thymic stroma and thymocytes by HIV [110, 111], the atrophy of the thymus in HIV infected subjects, which is similar to the age related "thymic involution" [112], and may be related to thymosuppressive effects of proinflammatory cytokines (like IL-6) (e.g., by inducing apoptosis of immature thymocytes) [113, 114]. In addition, immune activation and inflammation are thought to cause fibrosis of the lymphatic tissue (i.e., collagen deposition), therefore damaging its architecture and preventing normal T-cell homeostasis [115, 116]. HIV infected subjects are therefore characterized by a general decline of T-cell renewal capacities. Naïve T-cell numbers decrease during HIV-1 infection [117], in contrast to $CD28^{-}$/CD57+ cells, which accumulate in the CD4+ and particularly CD8+ T-cell compartments [67, 118]. Telomere length is significantly shortened in the CD8+ lymphocyte population of HIV-1 infected patients [119, 120], which may relate to the decreased proliferative capacity reported in this population [121]. These changes, together with alterations in cytokine secretion (e.g., decreased IL-2 production) [122] and increased susceptibility to activation induced cell death [39], reflect a general shift of the T-cell population towards increasingly differentiated and senescent cells [123], the likely consequence of HIV mediated systemic immune activation.

The precise mechanisms involved in the decline of regenerative capacity are still not completely understood, however the following hypothesis may be proposed. In order to supply new T-cells (to replace senescent, apoptosed and infected cells), precursors in the bone-marrow and in the thymus are mobilized. However, due to the

limitation in primary immune resources, this process may deteriorate with persistent activation and inflammation, resulting in the exhaustion of these resources. With the gradual decline of the T-cell renewal capacities, the naive T-cell pool is not replenished, and is therefore unable to continually replace old exhausted CD8+ T-cell clones and depleted CD4+ T-cells in HIV infected individuals, so that highly differentiated, oligoclonal and senescent antigen-experienced cell populations accumulate to fill the immunological space, reflecting the maintenance of homeostasis in the context of inadequate regenerative capacity.

As this occurs, the fragile balance between functional HIV-specific CD8+ T-cell activity and ongoing HIV-1 replication is broken. Uncontrolled viral replication rapidly depletes what is left of the CD4+ T-cell population, leading to immune collapse. The pace of this process may vary depending on the intrinsic pathogenicity of the virus, host genetic factors and also environmental factors. For instance, less pathogenic viruses (such as those with attenuating Nef mutations) are more readily controlled and are associated with clinical non-progression [124]. In the same line, age seems also be an important positive factor of HIV disease progression among HIV infected individuals [125, 126], possibly reflecting the impact of HIV-1 on an already ageing immune system.

5 Parallel with Age

5.1 The Immune Risk Phenotype

A comparison of the immunological changes observed in HIV-1 infected individuals with those accumulated with age in the HIV-1 uninfected elderly shows actually remarkable similarities summarized in Table 1 [123]. During ageing, a reduction in T-cell renewal together with a progressive enrichment of terminally differentiated T-cells, thought to be the consequence of immune activation over a lifetime, translate into a general decline of the immune system, gradually leading to immunosenescence [127]. Human ageing can result in clinical immunodeficiency, characterized by an increased incidence and/or rapid progression of many infectious diseases (e.g., influenza, pneumonia, meningitis, sepsis, varicella zoster virus, HIV) and possibly cancers. This leads to increased morbidity and mortality [128, 129]. The immunogerontologists have defined the “immune risk phenotype” (IRP), which regroups a cluster of immune measures that are predictive of early all-cause mortality in elderly persons [130]. Studies performed in the elderly revealed that the most significant factor of the IRP is the inverted CD4:CD8 ratio [130–134]. Moreover, longitudinal studies suggest that increased numbers of CD28-/CD57+ T-cells, poor T-cell proliferation, as well as seropositivity for CMV are part of the IRP [135, 136]. Interestingly, all these factors are features of HIV-1 infection.

Of note, CMV infection may hold a particular role of in HIV pathogenesis. CMV infection is extremely common in HIV-1 infected individuals, and CMV seropositive subjects generally experience more rapid HIV disease progression

Table 1 Similarities between HIV-1 infection and human ageing

Characteristics usually associated with human ageing		Also found in HIV-1 infection
Immunosenescence	Altered hematopoiesis	ref. 107–109
	Thymic involution and decreased output	ref. 86 & 112
	Reduced naïve T-cell numbers	ref. 117
	Inverted CD4:CD8 ratio	ref. 1
	Decreased IL-2 production by T-cells	ref. 122
	Reduced T-cell capacity to proliferate	ref. 121
	Shorter telomere length in CD8+ T-cells	ref. 119 &120
	Increased susceptibility to activation induced cell death	ref. 39
	Accumulation of highly differentiated T-cells	ref. 67 & 118
	Increased susceptibility to infections	ref. 1
Inflamm-ageing	High serum levels of IL-6/TNFα/IL-1β	ref. 41–45
	osteoporosis	ref. 145–148
	atherosclerosis	ref. 149
	neurocognitive deterioration	ref. 150–152
	frailty	ref. 153

than CMV seronegative subjects [137]. Interestingly, CMV infection in the elderly has also been associated with alterations in T-cell subsets that shows all the characteristics of replicative exhaustion [138, 139] and has also been associated with an increase in morbidity [136, 140]. Increasing evidence suggest that the anti-CMV response monopolizes a significant fraction of the whole T-cell repertoire [141] so that it might compromise the response to other antigens by shrinking the remaining T-cell repertoire and reducing T-cell diversity. In elderly individuals, CMV specific T-cells may comprise very high percentages of the CD8 population (up to 45%) [142, 143]. The accumulation of CMV specific T-cells has indeed been associated with reduced T-cell immunity against EBV infection [142] or after influenza vaccination [144]. Recurrent reactivation of CMV in HIV infected subjects may put further stress on the immune resources and thus could amplify the IRP of such patients.

5.2 Beyond Immunosenescence

The onset of a process of immunosenescence is not the only similarity between HIV-1 infection and human ageing: HIV-1 infected individuals present several alterations of physiological functions which usually characterize the individual of old age (Table 1). An increasing number of investigators have reported reduced bone mineral content and bone formation rate, along with osteoporosis in HIV-1 infected patients [145–148]. A study by cardiologists, endocrinologists, and HIV physicians also found more atherosclerosis in persons with HIV-1, with faster progression than in the general population [149]. In addition, HIV-1 infected individuals present

a variety of symptoms associated with the progressive deterioration of cognitive functions (e.g., memory loss, slower mental capacity, dementia) [150–152], that are usually related to old age. Last, recent work indicates that HIV-1 disease progression shows also a relationship with the onset of frailty [153], which corresponds to physiological alterations associated with advanced ageing (measured by unintentional weight loss, general feeling of exhaustion, weakness slow walking speed and low levels of physical activity) [154].

In view of these initial, yet fascinating observations, accelerated ageing in HIV-1 infection may therefore extend beyond the immune system to unanticipated facets of human health. The deterioration of several physiological functions in both HIV-1 infected individuals and HIV non infected elderly suggests parallel mechanisms of decline. Chronic immune activation and inflammation is likely to be again the cause of this systemic ageing of physiological functions. In response to tissue damage elicited by trauma or infection; proinflammatory cytokines like TNFα, IL-1β, and IL-6 are produced to initiate a complex cascade designed to destroy pathogens and activate tissue repair processes in order to return to the normal physiological state. However, the excessive production and/or accumulation of these mediators, as this happens during HIV-1 infection, may have adverse effects. TNFα, IL-1β, and IL-6 are thought to play a significant role in the process of ageing and are actually also found at higher concentrations in the blood of elderly [155, 156]. IL-6 in particular has been directly associated with the development of age related disorders including osteoporosis, cognitive decline and frailty symptoms [157–160]. Recurrent reports associate increased plasma levels of both TNFα and IL-1β in the elderly with atherosclerosis [161, 162]. In addition, a direct role of these cytokines is suspected in neuronal injury and neurocognitive deterioration [163, 164], possibly through the induction of large amounts of nitric oxide [165, 166], thus conducting to oxidative stress related damage [167]. Interestingly, links between CMV infection and atherosclerosis or frailty have also been recently established [168, 169].

This overall process can be referred as to “Inflamm-ageing” [170], that is the up-regulation of anti-stress responses and inflammatory cytokines. It is the consequence of the immune system ability to adapt to, and counteract, the effects of a variety of stressors. Paradoxically, it represents the main determinant of the most common age-related diseases and a major determinant of the ageing rate [171].

5.3 HIV-1 Infection: A Model of Accelerated Immunosenescence

In this part, we summarize the links between the different parts described above and propose a simplified model of HIV pathogenesis, which integrates its 3 main aspects, i.e., the massive depletion of CD4+ T-cells, the paradoxical immune activation and the accelerated process of immunosenescence (see Fig. 2).

A fundamental event in the HIV-1 pathogenesis is the infection of the CD4+ T-cell pool. During primary infection, HIV-1 is able to infect a large number of

CD4+ T-cells, in particular activated memory cells expressing CCR5. At this stage, the anti-HIV immunity is not mounted yet, so that viral replication and spreading remain uncontrolled. Viremia shoots up to reach peak levels, until the appearance of the immune response, in particular HIV specific CD8+ T-cells, which sees the end of the acute phase. However, the damage has been done: HIV-1 has been able to establish the premise of its latent reservoir, rooting itself in its host, and extensive viral replication has resulted in the massive depletion of CD4+ T-cells, in particular from mucosal lymphoid tissues. This has immediate consequences on the integrity of the mucosal surfaces, and microbial translocation ensues.

Considerable immune activation then takes place, which is multicausal and lasts throughout the course of the infection. First, the immune response against HIV-1 itself is activated, and aims at controlling the virus, despite persisting replication and emergence of variants that can escape both cellular and humoral responses. The immune system has also to cope with other persisting pathogens (like CMV), whose reactivation is enhanced by the substantial loss of CD4+ T-cells. HIV proteins can directly induce cellular activation. Last but not least, translocation of microbial products leads to systemic activation of lymphocytes and monocytes. As a conse-

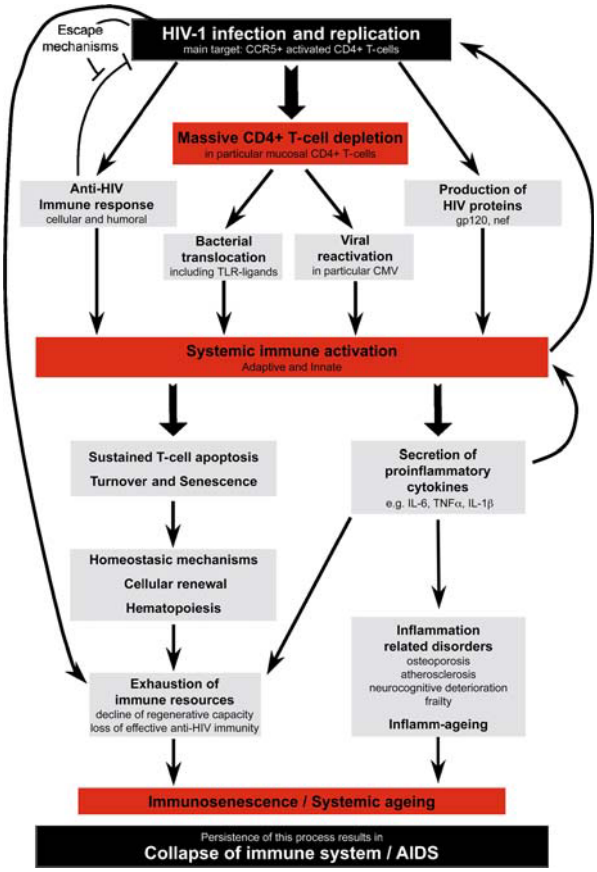

Fig. 2 HIV pathogenesis: a model of accelerated immunosenescence

quence, levels of proinflammatory cytokines increase notably. In addition, immune activation promotes HIV replication, thus establishing a viscous cycle.

Immune activation causes considerable cellular turnover, senescence and apoptosis, which represent a massive task for the immune system in terms of cellular renewal in order to maintain homeostasis. Overtime, the consequence is a progressive decline of regenerative capacities and the development of immunosenescence. In parallel, the elevated production of proinflammatory cytokines leads to the deterioration of a series of physiological functions. With the exhaustion of primary resources, naïve T-cells disappear and highly differentiated oligoclonal populations accumulate. Optimal anti-HIV immunity cannot be maintained and the CD4+ T-cell pool cannot be replenished, resulting in the collapse of the immune system ability to control pathogens, characterizing AIDS.

The development of immunosenescence is determined by the very physiological defense function of the immune system. Normal life is characterized by low grade, recurrent immune activation and inflammatory activity, which eventually leads to immunosenescence. Through the induction of persistent, sustained immune activation, HIV-1 infection may induce therefore an accelerated process of immunosenescence and systemic ageing. During this process, the immune system burns itself quickly, as the source of its combustion (i.e., the virus) cannot be put off.

6 Immune Activation and Anti-HIV Therapy

Taking into consideration the pivotal role of immune activation in HIV pathogenesis opens several possibilities of intervention or therapy to counteract the adverse effect of HIV-1 infection. These may be divided into 2 axes or strategies of treatment: upstream, in order to block or minimize immune activation and inflammation, or downstream, in order to restore or boost the regenerative capacity of the immune system.

6.1 Antiretroviral Therapy

The development of effective antiretroviral therapy or ART (which combines a series of inhibitors of the HIV replicative cycle) has proven to be decisive for the survival of millions of HIV infected patients, who can now live for years despite the infection [172]. ART remains the most successful therapy against AIDS to date. Through its potent inhibition of HIV replication, ART represents somehow the best “deactivator” of the immune system for HIV infected patients (see Fig. 1b). Although unexpected inflammatory disorders, known as immune restoration inflammatory syndrome can sometimes accompany the beginning of ART (due to augmentation of inflammation during immune reconstitution in immunocompromised HIV infected patients) [173], the abnormal activation and apoptosis observed during the course of the infection usually resolves with prolonged treatment with antiretroviral drugs, in parallel

to the plasma virus load reduction [174–176]. A similar decrease is observed for inflammation markers such as the proinflammatory cytokines TNFα and IL-6. Antigen specific stimulation is also strongly diminished, as seen with the rapid decline in the numbers of HIV specific CD8+ T-cells [177–179]. Eventually ART enables the reduction of naïve T-cell consumption and helps to restore their numbers. The indirect anti-inflammatory effect of ART may also play a role in the recovery of de novo thymic production. Thus antiretroviral drugs by blocking virus production reduce virus-driven immune activation and play a strong antiinflammatory role.

6.2 Prevention of Immune Activation

Other strategies are being developed to prevent immune activation and/or inflammation. For instance, treatment with the immunosuppressive drug cyclosporine A (which inhibits T-cell activation) has been tested: although significant increases in CD4+ T-cell counts were initially observed [180], the apparent benefit of cyclosporine A remains controversial [181]. Further along the activation pathway, one may attempt to inhibit the stimulating effect of bacterial products, translocated from the gut. For instance, antagonists of TLR-4, the receptor for LPS, could be good candidates in the context of HIV infection. One TLR-4 antagonist, Eritoran [182], has been tested in the context of septic shock where, it has diminished systemic inflammatory response by limiting the release of TNFα and IL-6 in mice [183]. On the same line, adapted inhibitors of proinflammatory cytokines could also deserve consideration as anti-HIV therapy. Several anti IL-1β, IL-6 or TNFα antibodies have been developed for use in humans and are currently being tested for their effect in inflammatory disorders like rheumatoid arthritis [184]. A recent study performed in primates revealed that long term caloric restriction could delay the process of immunosenescence [185]. Although the exact mechanisms remain to be understood, caloric restriction is likely to have a broad beneficial influence in lowering inflammatory and oxidative stress responses [155]. Last, one could also aim at reducing secondary challenges to the immune system. In particular, preventing CMV infection or reactivation may have a significant impact in delaying immunosenescence. In this context, the development of effective CMV vaccines may provide non negligible benefit.

6.3 Enhancement of Regenerative Capacity

Strategies to rejuvenate our immune resources are also being explored. Early trials with IL-2 resulted in marked expansion of circulating CD4+ T-cells, but the eventual benefit on disease progression has remained modest [186]. Recent data indicate that the adult thymus keeps some capacity to produce naive T-cells. As thymic activity has been demonstrated to be crucial for the full recovery of immune reactivity, namely for the reconstitution of the naive T-cell pool after T-cell-depleted

bone-marrow transplantation [187], reconstitution of the thymic microenvironment may be a critical factor for the success of strategies aiming at reversing immunosenescence. Thus immune interventions, which could lead to thymic "rejuvenation", represent a great interest both in the context of HIV infection and of ageing. Different strategies have been proposed based on the use of hormones or cytokines. Indeed, hormones influence both thymic maturation and thymic involution and it has been shown that castration of old mice resulted in the regrowth of the thymus at values found for younger animals [188]; in the same way, administration of synthetic inhibitors of luttinizing-hormone-releasing-hormone seem to restore thymic activity [189]. In elderly, it has been shown that treatment with growth hormone restores the cellularity of the thymus [190, 191]; and in the context of HIV infection, it results also in increased thymic mass and circulating naïve CD4 T-cells [192]. Age-associated thymic atrophy results also from defects in the thymic environment with lower amount of interleukin-7 (IL-7) available [85, 193, 194]. It can then be assumed that treatment by IL-7 could reverse thymic atrophy and could induce thymopoiesis, leading to more circulating naïve cells in the periphery. Of note, prolonged exposure of CD8+ T-cells to IL-7 or IL-15 can stimulate proliferation without differentiation or loss of telomere length [195]. Last, the potential of HSC transplantation may also be considered for therapy in HIV infection, since this can lead to the total reconstitution of the immune system.

7 Concluding Remarks

25 years of intense research on HIV pathogenesis have certainly taught us unanticipated lessons as to the depth of the relationship between a pathogen and its host, and the fragility of its equilibrium. HIV has developed several mechanisms in order to establish persistence, but it is really through its unique capacity to target a central element of the immune response, i.e., the CD4+ T-cells, that it can deregulate the integrity of its host immunity so efficiently. Nonetheless, this may be considered as imperfect, since as a consequence of infection, its host will undergo accelerated exhaustion and die, thus ending the virus life cycle. In contrast, CMV and EBV seem to have adapted to their host. These pathogens are seen as ancient viruses, which may have undergone a process of evolution overtime to become highly prevalent in humans. HIV may be seen as a very young virus in comparison. Research supports that a jump between species, from primates to humans, occurred during the 20th century, with SIV being the ancestor of HIV. Interestingly, the natural hosts of SIV, i.e., Sooty mangabeys and African green monkeys do not progress towards AIDS after SIV infection. Investigating the mechanisms of adaptation (e.g., that prevent systemic immune activation and therefore the onset of immunosenescence in these primates) could certainly help the design of effective strategies to fight HIV-1. A recent study has actually revealed that the Nef protein from nonpathogenic SIV strains as well as HIV-2 harbors a T-cell activation suppressing function (through down-modulation of the TCR complex), which was lost by HIV-1 [196].

In contrast to nonpathogenic SIV and primates, HIV-1 and humans may require further adaptation. Ironically, HIV-1 may lead to the accelerated ageing of its host because of its youth.

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Autoimmunity

Autoimmunity and Autoimmune Diseases in the Elderly

Ewa Bryl and Jacek M. Witkowski

Contents

Abstract: In the majority of papers dealing with immune system changes, higher prevalence of autoimmunity and autoimmune diseases among elderly people is stressed as one of the factors confirming the changes of immune system function in aging. This statement is repeated for a very long time and most of the authors treat it as a "stone-carved" truth. However, we will show below that, as we look into this problem in details, there are not so many diseases which appear in the elderly population more frequent to the younger ones. So, where is the problem and why is that so?

1 Autoimmunity Phenomenon in the Elderly Population

In order to discuss the topic, we first need precise definitions of autoimmunity, autoimmune diseases and their diagnosis. Classical definition of autoimmunity describes it as a hostile, improper reaction directed against autologous antigens. There are two major ways of checking such improper immune reaction existence in human body: one searches for autoreactive immune cells (mainly T-cells) and the second (but by far more popular) is focused on searching for autoantibodies. Looking for human autoreactive T-cells is limited to those possibly present in the peripheral blood, and it is not an easy task, mainly because of usual lack of identified autoantigen. Current MHC tetra- or pentamer technique facilitates the screening, but it still requires known aminoacid sequences in the tested, potentially antigenic peptide. Much easier way of looking for an autoimmune reaction is therefore to find the autoantibodies circulating in the serum of the individual that we suspect to develop such a reaction (or just for screening). One has to bear in mind that autoim-

J. M. Witkowski (✉) · E. Bryl
Department of Pathophysiology
Medical University of Gdańsk
Debinki 7
80-211 Gdańsk, Poland
E-mail: jawit@amg.gda.pl

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munity thus defined does not necessarily have to be overtly pathogenic, i.e., be a part of pathomechanism of any disease.

The definition of an autoimmune disease is not so clear, at least for some of them. As for all diseases, also the diagnosis of autoimmune diseases is made based on clinical symptoms (deviations from norm that describe the disease), usually defined by scientific committees for such diseases. For the majority (if not all) of autoimmune diseases diagnosis is based on detection and/or determination of the levels of certain autoantibodies (Table 1) which, together with other clinical symptoms finally define the disease.

In some diseases the self (auto-) antigen is well defined and these diseases are usually not causing diagnostic problems; the majority of them could be called organ-specific autoimmune diseases, indicating that they usually involve one organ or system of the body; there, the auto-antibodies detected will also be "organ-specific." In many cases the self antigen is not known and autoantibodies are used for diagnosis based on their more frequent appearance with combination of disease's symptoms and thus are not specific for type of disease. This is a case for a majority of autoimmune diseases called systemic autoimmune diseases.

The problem with such definitions is that autoantibodies are the end-product of improper immune system activity and not its beginning (so, ultimately, they cannot be considered the primary cause of an autoimmune disease), and the level (titer) of these antibodies may not be dependent on the actual strength of the immune reaction. Many of autoantibodies were reported to be increased in the healthy elderly population as compared to young ones (Table 1). However, the tendency of titer of various autoantibodies to increase with age is not uniform. Especially, so called organ-specific and nonspecific autoantibodies reveal different patterns. The organ specific autoantibodies increase in healthy people can be observed until 80–90 years of age, but the centenarians show lower prevalence of these autoantibodies [16, 50]. For example, the prevalence of anti-thyroglobulin and anti-parietal cell autoantibodies in centenarians had not differ from that recorded for 60 years old healthy subjects [16].

Quite different picture was observed for nonorgan specific antibodies, like anti-cardiolipin and anti-nuclear antigens, which constantly showed higher prevalence with advanced age, including centenarians [16, 49].

In one of the studies cited above, the anti-nuclear antibodies (ANA) were found in sera of elderly people (mean age 80 years), with frequencies 31.3% [49], but not correlation was found with age or sex. The presence of higher frequency of ANA in the sera of elderly people seems rather a common finding, which seems to be indeed aging-dependent, as no changes in ANA frequency were found in healthy people between age 20 and 60 [90]. The latter authors raised a valid methodological point concerning their and similar studies, i.e., the titer of autoantibody, which should be considered positive; different dilutions gave different positivity rates, for example changed the frequency of positive ANA in the same group from 31.7% to 3.33% for 1:40 and 1:320 dilutions, respectively [90]. These observations resulted in a change regarding what titer of ANA should be considered "normal"; thus, anything below 1:40 for 20-30 years olds and below 1:80 for people above 60 years old is now

Table 1 Autoantibodies in elderly population

Autoimmune disease	Diagnostic autoantibodies	Specificity for disease	Present in asymptomatic[a] elderly	Proportion of healthy elderly people having the autoantibody [source]
Autoimmune thyroiditis	Anti-thyroglobulin	High	Yes	9.2% [88] 22.2% (>80 years old women) 11.4% of centenarians [4]
Autoimmune Hashimoto thyroiditis	Anti-thyroid peroxidase	High		15.3% [88] 25% (>80 years old women) 10% of centenarians [4]
	Anti-thyroglobulin or anti-thyroid peroxidase		Yes	4.16% vs.10.4% (centenarians vs. elderly (72–93) [48] 14.6% vs. 15.6% (older women vs. middle-aged women) [33] 4.3% vs. 0 (older men vs. middle-aged men) [33] Age-dependent increase in prevalence from 7 to 85 years, in centenarians no different than of age 50 [50]
Graves disease	Anti-thyrotropin receptor	High	No	ND (no data found)
Autoimmune gastritis (Pernicious Anemia)	Anti-parietal cell antibodies (H/K ATPase)	High (60–90% of patients)	Yes	36% of healthy people (mean age 48.2 years) [47], 15% in aged more than 85 years [70], 18.6% of centenarians [4]
	Anti-intrinsic factor (IgG)	High (50–70% of patients)	ND	
Primary biliary cirrhosis	Anti-mitochondrial antibody (AMA)	High	Yes	5% in aged more than 85 years [70]
Multiple sclerosis	Anti-myelin basic protein antibody Anti-myelin oligodendrocyte glycoprotein antibody	High	ND	

Table 1 (continued)

Autoimmune disease	Diagnostic autoantibodies	Specificity for disease	Present in asymptomatic[a] elderly	Proportion of healthy elderly people having the autoantibody [source]
Mysthenia gravis	Anti-AchRantibodies	High	No	
	Anti-titin antibodies	reported in elderly	No	[110]
	Anti-Ryodine antibody	onset	No	
Sjogren Syndrome	Anti-nuclear antibodies	High	Yes	34% [58]
	Anti-Ro SSA, Anti-La SSB	High (but only 35% patients)		
	Anti-smooth muscle antibodies (SMA)	62% of patients		
Goodpasture syndrome	Anti-collagen type IV α3-chain antibody	High	Yes	
Pemphigus vulgaris	Anti-Desmoglein 3	High	No	[93]
Bullous pemphigoid	Anti-basement membrane zone antibodies	High	Yes	19% [32]
Pemphigus foliaceus	Anti-Desmoglein 1	High	ND	
Rheumatoid arthritis	RF IgM	Low	Yes	26.6% of centenarians [4]
	RF IgG or IgA	High		
	Anti-CCP (anti-cyclic citrullinated peptide)	Moderate		
	Anti-keratin antibody (AKA)			
SLE	ANA	Low	Yes	10% in aged more than 85 years [70]
	dsDNA antibody	Moderate	Yes	14.3% of centenarians [4]
	Anti-phospholipid antibodies	20–40% of SLE	Yes	54.3% centenarians IgG and 8.6% IgM anti-beta 2 glycoprotein I antibodies [54]
	Anti-cardiolipin antibodies (IgG; ACL)	20–30% of SLE	Yes	20.7% IgG and 2.59% IgM [54]

Table 1 (continued)

Autoimmune disease	Diagnostic autoantibodies	Specificity for disease	Present in asymptomatic[a] elderly	Proportion of healthy elderly people having the autoantibody [source]
Autoimmune hepatitis (AIH) Type 1	Smooth muscle antibodies (SMA)	High	No	
	Anti-nuclear antibody (ANA)	Low	Yes	
Type 2	Antibodies to liver/kidney microsome type 1 (anti-LKM1)	High		
Type 1 and 2	Asialoglycoprotein receptor	Moderate	ND	
Myositis, Dermatomyositis	Aminoacyl-tRNA histidyl synthethase	High	No	
Systemic sclerosis	Topoisomerase-I (Scl-70)	High	No	

[a] Without autoimmune disease symptoms.

considered ok. However, even with this change, the majority of reports agree with higher frequency of ANA in healthy elderly peoples' sera.

In another study, the overall prevalence of these autoantibodies rose steadily with age, particularly in females, until the seventh decade, and after the age of 75 there was a sharp fall flowed by a steep rise in very old subjects for ANA [37].

Interestingly, more controversial data were obtained for antidouble stranded (ds)DNA antibodies. Some reports show about 14% of elderly people aged 80+ years old positive for these antibodies [49], but the others did not find anti-dsDNA antibodies (found in the sera of people suffering from various autoimmune diseases, notably the SLE) in any of the examined age groups including people over 80 [16]. About 17% of the elderly sera were also found to contain anti-single stranded (ss)DNA antibodies, and the majority of the elderly burdened with these antibodies were aged more than 81 years old [49].

Rather uniform data exist for anti-cardiolipin antibodies—the majority of data showed about 50% appearance in the serum of apparently healthy elderly people [16, 49]. While some autoantibodies had a tendency to appear together (e.g., dsDNA autoantibody always were detected in the presence of other autoantibodies), in half of individuals the anti-cardiolipin autoantibodies appeared alone [49].

The similar phenomenon was described for rheumatic factor (RF) an autoantibody directed against immunoglobulins. The proportion of elderly people with positive IgM RF reached 26.6% and for IgA RF 18.7%, supporting the idea about nonorgan specific autoantibodies increased in the elderly [4].

Finally, when the organ- and nonorgan-specific antibodies were sought in healthy Danish centenarians, out of 79% of them that had autoantibodies the majority of them were nonorgan specific (in 47% of subjects), whereas organ-specific antibodies only were found in 8% of centenarians [4].

These findings are parallel to changes in the cellular immunity described by others, where T-cell function measured by different means was frequently reported to be impaired in healthy elderly people, but not in centenarians, e.g. [54, 59, 67]. Similar observation was made for the NK activity, but the age above 80 was already the border for high NK activity of healthy people [56].

The above findings tell that there is a tendency for certain autoantibodies to increase in titer or prevalence in the otherwise healthy elderly population. Unfortunately, these observations were sufficient for many authors to describe old age as autoimmune disease prone and to state that the frequency of autoimmunity meant as autoimmune diseases is increased in the elderly cohort.

Another question which seems important is: should all autoantibodies detected in the person's serum be treated the same way? Are they all telling us about the same? What does it clinically mean that more autoantibodies appear in the plasma of elderly people? There is a possibility that they are not pathological, but serve as regulators of the immune reaction—reviving the old concept of immunoglobulin network [82, 89]. Perhaps they control each other, which in the known case of decreased central suppression from thymus observed in the elderly due to thymic involution should be considered an adaptation process. Do we have answers for the questions posed that way? We believe that some answers are already possible;

below we show an example that in our opinion tells that not every appearance of autoantibodies should be treated as a manifestation of ongoing or imminent autoimmune disease.

Our example are the organ-specific versus not-organ-specific autoantibodies. We are temped to put below the hypothesis about the possible different reasons for their appearance.

Organ specific autoantibodies are in majority connected with a more or less defined pathology of specific organ and many of them are directed against surface markers of "attacked" cells— like anti-acetylocholine receptor antibody, anti-TSH antibody, etc. Thus, essentially, the precondition for their appearance would be a mistaken recognition of self as alien or lack of proper regulation (tolerizing 'switch-off of the reactive clones) due to less suppression. When we consider such a possibility, the thymus derived natural regulatory $CD4^+$ T-cells (or—in fact—decreased function of these) are the first to come to mind. Decreased thymic function in the elderly is well-described, meaning both—fewer new emigrants from the thymus in the elderly [111] and less humoral activity [59]. The removal of thymus in young animals was shown to lead to the development of autoimmunity, and especially of organ-specific autoimmune diseases [9, 80]. The reason for this phenomenon could be that after thymectomy fewer $CD4^+CD25^+$ regulatory T-cells appeared in the peripheral circulation of studied animals [7, 53]. If the animal models can be extended to humans, it would lead to conclusion that the decreased function of the thymus in the elderly would manifest not only as a generalized lower output of new T-cells, but also as relatively decreased numbers (and function) of the "natural Tregs" [20]. *Very few* publications so far deal with this problem mainly because of problems with Tregs definition, therefore published data are contradictory, showing the relatively decreased numbers/proportions or function of natural $CD4^+CD25^+$ T-cells in peripheral blood of elderly people [97, 100], whereas the opposite finding can be also found [31, 96]. Please see the chapters by P. Moss and J. Shimizu in this volume for a current update on the regulatory T-cells in the elderly. Summarizing, it looks possible that organ-specific autoimmunity can be in part a result of less thymic function and thus derailed T-cell dependent regulation in elderly people.

The autoantigens which are directed against the intracellular epitopes present in the cytoplasm, mitochondria or nucleus seem to have a different genesis and meaning. On one hand, intracellular epitopes (especially the ones containing lipids or fragments of DNA in addition to the peptide stretches) are sequestered inside the living cells and physiologically unavailable for the recognition and reaction by the immune system. On the other, even if generated, such an autoantibody could not enter the living, intact cells to react with 'its' antigen! So, are these autoantibodies (like for example the anti-nuclear and anti-DNA antibodies) the result of a process different than autoimmunity?

The necessary step in the process of triggering the anti-nuclear or anti-DNA antibodies is the release of antigen or its epitopic fragments from the cells, in order to be picked up by the APCs at the beginning of the autoimmune reaction. It implies the destruction of own cells as a start of that process. The two major processes of cellular destruction, leading to the generation of cellular fragments or debris that may

become the source of—previously unavailable, hence not tolerized—autoantigens are the apoptosis and necrosis. Many data are confirming the greater susceptibility of lymphocytes to undergo apoptosis if the source of lymphocytes is a peripheral blood of elderly people (e.g., [41, 46, 68]). The same is apparently true for many other human cells and tissues, including notably the skeletal and heart myocytes, nerve cells, and neutrophils [21, 27–29, 35, 107, 113]. Lymphocytes seem to be more resistant to apoptosis than other cell types, so it is very likely that apoptosis rate is greater in tissues of elderly people, providing larger quantities of apoptotic bodies as the source of many autoantigens, some of which can—at least in theory—start the autoimmune reaction.

Destruction of the cells leading to the liberation of their internal contents (including the putative autoantigens to be picked up by the APCs) can be also due to the process of necrosis. This type of cellular death can be a result of many causes. It was found by numerous authors that the necrotic death of various cell types is more frequent in the aged humans, possibly due to increased fragility of the cellular membranes, lower resistance to stress, mechanical and chemical agents, etc. [40, 55, 62, 94]. One interesting possibility is that increased necrotic destruction of the cells in an elderly individual is due to lower immunity to viral infections, and the lytic activity of viruses invading increased numbers of cells.

More dead cells in the body of elderly people should cause increased level of inflammatory process, which is a physiological response to the cells' damage. Majority of available data support that hypothesis, showing that the immune system of elderly people is acting more proinflammatory, and the balance is shifted towards (subclinical) inflammation even in apparently healthy elderly. This is the basis of the term inflamm-aging coined by Franceschi et al. [26]. Available data show increased concentrations of proinflammatory markers like IL-6 and neopterin in the sera of elderly healthy people [17, 57]. Since neopterin is a product of activated monocytes, these findings indicate rather the in vivo activation of monocytes, especially that the numbers of peripheral blood monocytes are not different between young, middle aged and elderly people [13]. In the light of these facts, maybe we should look at the antinuclear (and possibly also other antiintracellular antigen) antibodies as the result of "normal" reaction to the cells' destruction. Combined with less immune complex clearance due to decreased expression of Fc receptors on phagocytic cells of the elderly or decreased phagocytosis of the immune complexes by these cells [12, 15] it could give as a result the observed increased levels of autoantibodies.

Increased inflammation can also be a result of improper innate immune response. Majority of data showed that monocytes and macrophages isolated from elderly people, both unstimulated and stimulated in vitro, produce more proinflammatory cytokines (IL-1β, TNF, IL-6, and IFN-γ, detected both by measurements of secreted cytokines and intracellular in monocytes) than the "young" ones [13, 24, 72, 75, 61]. The existence of more pro-inflammatory status of elderly people was demonstrated also in an in vivo experiment, when the bolus of endotoxin was given and the acute phase response was compared in age groups. The elderly people showed an initial hyperreactivity (measured by higher and faster increase of TNF concentration), higher proinflammatory activity and prolonged fever response [44]. Also, surgical

stress caused higher TNF and IL-6 production in elderly people than in the younger group, who underwent the same surgery process [63]. All the abovementioned facts seem to indicate for more monocytes activation in the elderly people in vivo, and increased proportion of subpopulation of activated monocytes in peripheral blood of elderly people [77]. The reasons are not fully explained, but changed expression and/or function of the "first danger sensors" of innate immunity, including the expression levels and functions of toll-like receptors (TLRs) and triggered receptor expressed on myeoloid cells (TREM-1) should be considered as a possibility. However, limited data so far did not seem to demonstrate the increased expression or function of TLRs (reviewed in [102] or TREM1 signaling [25]. Thus, high mobility box protein 1 (HMGB1, secreted from monocytes and macrophages or released from necrotic cells) emerged as a candidate target responsible for more active status of monocytes in the elderly.

HMGB1 stimulates monocytes and macrophages and acts through the receptor called RAGE (receptor for advanced glycation end products); that indicates that the function of the RAGE/HMGB1 system may be enhanced in the elderly people with increased levels of circulating AGE products. Activity of the RAGE/ HMGB1 results in increased production of proinflammatory mediators. It also acts on endothelial cells—induces VCAM-1, ICAM-1 and RAGE expression and stimulates secretion of TNF, IL-8, MCP-1 and PAI-1 (plasminogen activator inhibitor -1) and tissue plasminogen activator. HMGB1 acts also on enterocytes, increases the permeability of enterocytic monolayers (epithelia) and bacterial translocation to lymph nodes (mice in vivo). One of the theories about increased inflammation in elderly people suggests larger "bacterial load" in intestine being a reason for the process [105]. If so, the increased activity of HMGB1 could be a possible connection of these two concepts leading to more permeability of intestinal wall for intestinal commensal bacteria and causing a pro-inflammatory status of elderly people. A recent finding demonstrates that, at least in a mouse model, HMGB1 may act as an adjuvant increasing the production of autoantibodies [76].

Summarizing, the processes leading to the accumulation of autoantibodies (especially those directed against the intracellular antigens) in the healthy elderly people could be depicted as in the Fig. 1.

2 Autoimmune Diseases in Elderly Population

According to many, even current, positions on the subject, the autoimmune diseases are more frequent in the elderly. However, this notion is too general and requires more detail in order to be understood precisely. First of all, we need to distinguish the two important parameters: one would be the cumulated prevalence of any (or all) autoimmune disease(s) in the elderly (above 65 years of age) cohort, and the other, the proportion (or incidence) of actual NEW cases in each age group.

Reported higher proportion of elderly people suffering from *manifest* autoimmune diseases could be due to the nature of these disorders, which could be characterized

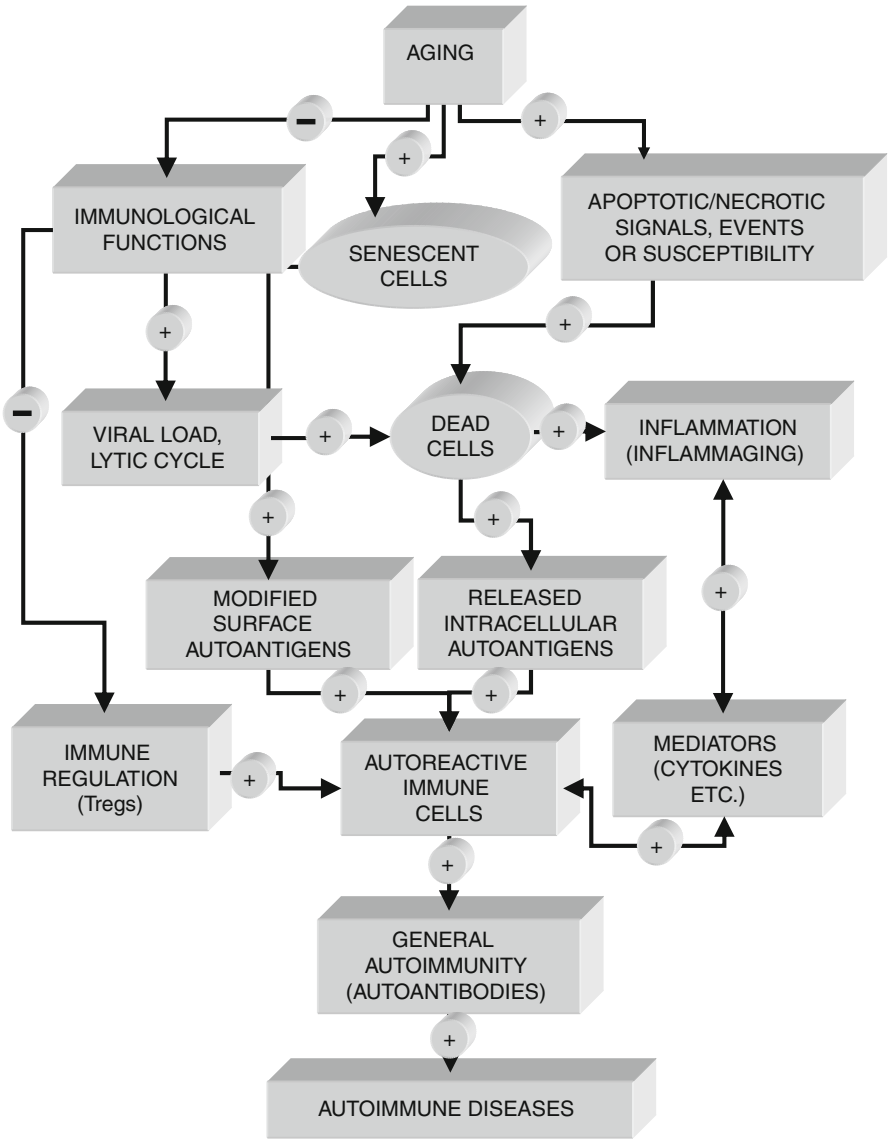

Fig. 1 How aging leads to increased autoimmunity. +: increased number, proportion, function; stimulation-: decreased number, proportion; function; inhibition

as chronic, incurable and nonlethal. If we remember, that—once diagnosed—an autoimmune disease "stays" with the patient for the rest of person's life. Thus, it is a very easy way to accumulate the percentage of sufferers of autoimmune diseases with increasing age of population. One can even predict, that—in the future—more and more elderly people would have been diagnosed with autoimmune diseases which, at least in part, will be dependent on modern medicine being more effective

in diagnosing and treating of these diseases and thus participating to the longer life of the patients. Modern medicine however, with all its drugs can only slow down the progress of the diseases or modify their course, but definitely not cure them, at least for now.

On the other hand as we look into the available statistics of "first diagnosis" of autoimmune diseases for adults, many of them start at 20–30 years of age and have the highest incidence between 40 and 50 years of age and NOT in advanced age (after 65 years, Table 2). A good example of such a case is rheumatoid arthritis (RA), considered for many years a disease of elderly people, mainly because of the fact of late diagnosis and the diagnosis at late stage, which was and still is prevalent among people aged more than 60 years. Based on a very well defined study from Rochester, Minnesota, USA [22] the conclusion can be drawn that in the cohort of people born in 1880 and 1890 the RA incidence was highest in women above 80 years old, and in the cohort born in 1910 or later—the median age of diagnosis was about 50–60 years old. Along with that tendency one can even be tempted to think that the highest incidence of RA in modern times should occur even earlier! However, this would be probably jumping to conclusion, because the definition and recognition of RA changed during last 60 years, and at the age of 50 people born in 1890 could not be diagnosed properly; the authors of cited paper themselves described that the mean age of incidence remained stable over the 40 years of observation time [22]. An interesting observation was also made for gender differences, with women aged 35 to 44 years having a higher rate of RA incidence than men from the same age group, while the disease incidence for both sexes at age 75–84 was comparable [22]. Different RA incidence dynamics were also reported in the longitudinal studies reported by these authors—the incidence in women rose until age 55–64 after which it steadily declined, but for men it started very low at the age 18–34, but later the incidence progressively increased until the oldest group observed (> 85 years old), when it decreased dramatically. One of the conclusions of the same report is also that the mean age at diagnosis is lower for women than for men [22].

The drawback which should be taken into consideration when considering these data is that there is no available information on how many patients were hospitalized and how many were in Outpatients Clinics Care; in our opinion lack of this information or it not being considered by the authors of the study may change the recorded age at diagnose. The mean age of hospitalized RA patients should be higher than mean age of RA patients in Outpatients Clinics; however, since no direct data were published about this problem, the indirect confirmation can be the RA frequencies in patients hospitalized because of pneumonia, where 62% of all hospitalized RA patients were above 65 years old [109]. It is easy to imagine that if the data for that study were obtained from hospitals, the reported mean age of RA patients at diagnosis would be higher, while the Outpatients Clinics see on average patients of lower age and often are the source of the first diagnosis, also for the autoimmune diseases. The example above shows how biased may be the estimation of actual ages at diagnosis, incidences in relation to age even for an apparently very well known and epidemiologically described disease as the RA.

Table 2 Epidemiological data for autoimmune diseases in adult population

Autoimmune disease	Mean age of onset (range)	Age of highest incidence of disease (years old)	Percent of new onsets before age 65	Percent of new onset after age 65	References
Rheumatoid arthritis	46 years (France)	55–64 F			
	47.3 years (Germany)	70–74 M	70–80%	20–30%	[78]
	48 years (Spain)	(Minnseota, USA)			
		40–60 (Spain)			
Myasthenia gravis	Around 20	About 20 F	80–94%	6–20%	[36, 65]
	Divided into 2 age groups	2 peaks for women:	41%	59%	[79]
		30–39 and 70–79,	40%[a]	60%[a]	[85]
		for men 60–69	70%	30%	[52]
Multiple sclerosis	30 (16–49)	25–30	95.3%[a]	4.7% (2.7–12%)[a]	[95]
	30.79 (14–53)	30–39			[106] [69]
Autoimmune thyroid diseases:					
Hashimoto's thyroiditis	36.1 (8–77)				[3], [10]
Graves' disease	42.2 (8.2–87.2)		65%[b]	35%[b]	
Sjogren syndrome -primary	52.9 years (15–87)	Women, middle age			[58]
Systemic lupus erythematosus:					
Caucasian	47 years	50–54 F	Majority[a]	Rare (714 cases in	[84]
		70–74 M		literature) [11][a]	[112]
Diverse population	40.9 years (18.5–81.6)	40–49 F and 50–59 F			[38]
Mixed connective tissue disease	52±17 years		Majority	Rare	[42]
Dermatomyositis	41 ±10 years		Majority	Rare	[42]
Polymyositis	49±10 years				
Autoimmune hepatitis (AIH) Type 1:					
Caucasian	48 years (18–83)	10–30 and 40–50	77%	23%	[2, 19]
			74%	26%	
Not European caucasian	30 years (12–58)		100%	0%	[114]
Primary biliary cirrhosis caucasian	59 years (34–78)	40–50	61%	39%	[34, 60]
Bullous pemphigoid	Affects elderly individuals				
Systemic sclerosis	38 years	33–43 years	Majority	Rare	[6]
	48±15 years				
Pernicious anemia	60 years (23–90)				[5]

[a] Report puts the border of late-onset at age 50 years for SLE, myasthenia gravis, multiple sclerosis.

[b] Report puts the border of late-onset at age 40 years for Graves' disease.

To support the notion of lack of correlation between rising incidence of RA and age of patients, a long-term observation which reported only the incidences of RA in the cohorts aged above 65 years, clearly showed that these incidences were decreasing with advancing old age, being the highest for patients aged 65–75 and lowest for those aged 85–96 years [81].

Interesting data for the same disease were obtained in Netherlands, where the age of onset of RA was compared between sporadic and familial RA defined by presence of at least two siblings fulfilling the ACR criteria [71]. The authors did not find any differences in age at onset between familial and sporadic RA groups when *the whole population* was analyzed. However, again the situation was different if women and men groups were analyzed separately. In the examined women population the percentage of first diagnoses being set before the patients reached the age of 60 contains 77% cases for sporadic and 81% for familial RA in women cohort, with a peak about 60 years of age for familial RA [71]. Similar analysis in men cohort revealed that 60% of sporadic RA diagnosis was done before or at 60 years of age, while as many as 88% of diagnoses for familial RA were set before the age of 60, with the highest frequency in the middle aged cohort (40, 45–60 years old men) [71]. The earlier onset of familial RA seems to be logical as RA is thought to be dependent in a large extend on genetic background, but it was found only in men population. The later onset for familial RA in women group is rather unexpected finding and is difficult to explain.

Apart from cited above, limited epidemiological studies exist for the prevalence of RA in outpatient practice. Based on the recent Dutch data the mean age of RA patients was about 56 years, and the age at first diagnosis was younger [104]. Unfortunately that publication did not mention the age at diagnosis.

The patients' age at disease (RA) onset can be also influenced by different diagnostic criteria applied and not always "clean" clinical picture at the beginning. American College of Rheumatology (ACR) criteria for RA diagnosis allow to define the cases for epidemiological studies, but it was observed that in individual cases, even if person had not fulfilled ACR criteria during the first visit, they were fulfilled (and the patient diagnosed with RA) on second or next visit 3, 6 or 12 months later [23, 64]. Obviously, such a patient was having the incipient RA already at the first visit and the date of it plus patient's age at the first reported symptoms should be recorded as the age of onset and age of diagnosis, respectively. One of these relatively late criteria is the existence of detectable bone erosions, and many rheumatologists are at opinion that diagnosis at that stage is quite late and the damage was already done; i.e., the disease had to be lasting for at least many months prior to the appearance of this symptom! Recently, the necessity for early diagnosis is not longer as a matter of discussion (dysputable), and many programmes have this as a target [18, 101].

Also our own data obtained after 7 years of observations revealed that among 74 RA patients from local Outpatient Rheumatologic Clinic the age of onset for the majority of patients was between 40 and 60 years (Fig. 2). In the light of these facts (including our own observations), it is hard to confirm the opinion that RA is a disease of elderly people. Rather, it becomes the disease of middle-aged people that,

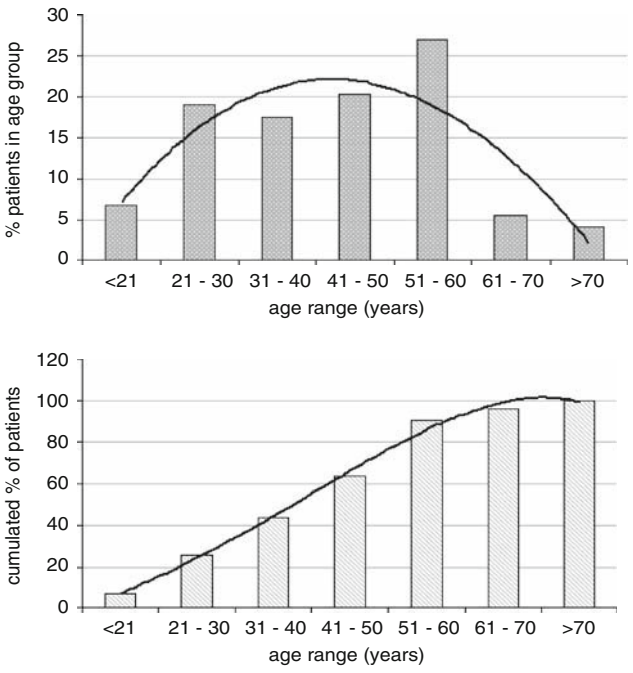

Fig. 2 Incidence (*upper panel*) and cumulative proportion of patients in consecutive age groups. Graphs show frequency histograms (*bars*) and polynomial trend lines; N=74

because of (still) lack of complete knowledge regarding its causes and pathomechanism, and therefore lack of really successful therapy, remains with the patients to the end of their life and, with increasing average lifespan in the western population, boosts the statistics of its occurrence in the 65+ year olds.

However, the possibility of late onset of RA is a fact, and nowadays, two forms of adult RA are recognized and defined by age of onset of disease—early onset RA—onset before 65 years old (about 70–80% cases) and late onset RA—onset at age above 65 years old (about 20–30% cases) [99, 103]. The studies comparing the outcome of these two forms of disease were performed and in many of them the outcome was more favorable for late-onset of RA measured by fewer and less aggressive joint erosions, or easier remission [99]. Clinical differences reported include more frequent shoulder involvement, less classical hand deformities, interstitial lung disease and Sjogren's syndrome, but more frequent weight loss, myalgia, lymphadenopathy and polymyalgia rheumatica –like syndrome in the late onset RA [8, 98]. The laboratory data were also different with the late onset RA characterized by lower rheumatoid factor (RF) frequency, less specific autoantibodies like ANA, anti-SSA/Ro and anti-SSB/La, but elevated ESR and C-reactive protein [98]. The differences between early and late onset RA patients are variable throughout various human populations; for example less differences were found for Greek population [66] and for North American Caucasians, but even in those studies despite more frequent methotrexate usage for late onset of RA, the doses were significantly lower and the number of DMARDs used in the elderly group with late onset was also significantly lower, indicating lower clinical activity of this form of the disease [99].

One recent study showed that late onset of symptoms of polyarthritis in age group above 50 years old, favors more erosions within 1-year of observation, the difference being 51% versus 71% between groups 50–69 years and more than 70 years old [14]. However, this could be caused by milder symptoms of arthritis in older group and later diagnosis, or less aggressive treatment of older patients. Thus, based on the majority of clinical observation of late-onset RA patients, its clinical course is generally milder and easier to achieve remission.

The data comparing the immune system in elderly RA patients with new onset of disease with elderly healthy people are limited. Based on existing publications, for example the telomere length in $CD4^+$ T-cells in both elderly groups are comparable [43], but information is lacking if these were elderly with new onset of disease, or patients who had long RA history. A recent paper showed that shorter telomere length in lymphocytes isolated from peripheral blood of RA patients is not dependent on duration of disease, nor disease activity [87]. The similar story is for measuring TRECs content in $CD4^+$ T-cells, which is lower number in elderly RA patients [43].

Rheumatoid arthritis is even more interesting and relevant to the topic of this volume when we consider that the studies of the properties of the lymphocytes (especially of the $CD4^+$ T-cells) isolated from RA patients aged 20–30 years old and examined less than 1-year from disease onset, showed a profound changes in phenotype and behavior (especially in vitro proliferation dynamics), resulting in the patients' cells being similar to the $CD4^+$ lymphocytes isolated from healthy 60–70 years old people [108]and own unpublished data. So, the picture which is quite opposite to the established paradigm is emerging: not only RA is NOT a de facto disease of elderly people, but moreover, its early adult onset is associated with the accelerated immunosenescence of $CD4^+$ T-cells. RA starting in younger age is usually more aggressive and clinical changes are going faster, unless the disease is treated more aggressively. It is possible to imagine that early start of the disease in young individuals, when the immune system is more vigorous, could result in more aggressive form of arthritis than in case of RA onset at or above 65 years of age. In the latter case, the course of disease is described as milder possibly because of less vigorous immune system, leading to less tissue damage.

Obviously, rheumatoid arthritis is by far not the only autoimmune disease that can be considered more deeply in relation to the patients' age and to the "functional age" of their immune systems. Few other notable examples (the number of which must be kept low due to the space limitations in the volume) are multiple sclerosis (MS) and myasthenia gravis (MG).

A peak onset age of MS about 30 years is quite typical worldwide [106], but the characteristics of immune system of these young people is already comparable to that of healthy elderly people. Specifically, lymphocytes isolated from MS patients showed shorter telomeres and higher proportion of $CD4^+CD28^-$ cells in the peripheral blood [91, 92], which is in very similar to the findings in young RA patients and healthy elderly people [30, 43]. Also, the levels of TRECs-expressing $CD4^+$ and $CD8^+$ T-cells (early thymic emigrants) were significantly decreased in MS patients, and matched those of healthy individuals who were 30 years older [39], which is again a similar to RA findings [43].

However, so called late-onset MS was also reported, but the age limit distinguishing the early- and late-onset disease was set at 50 years old; thus, also the late onset MS cannot be considered the disease typical for elderly. The report indicates the prevalence of late-onset between 4% and 9.6% [51]. The course of the disease is often primarily progressive and pyramidal or cerebellar involvement is observed in the majority of MS patients with onset above 50 years old. Late onset of MS was also associated with a faster progression to disability and more atypical forms of the disease; here, the differentiation can be difficult due to accompanying diseases including the most frequent cerebro-spinal vascular syndrome, and hypertension-related disorders [51]. Despite the fact that the set limit of 50 years does not in fact distinguish between young and elderly MS patients, the percentage of elderly people with MS could increase, mainly due to longer life of patients, similar to the situation observed for RA.

Considering MG studies performed across last 30 years – they report an increasing age of onset. The original reports (from late 70-ties) describe MG as a rare disease, occurring mostly in young women around 20 years old [45]. Within the next 20 years, the observed age of onset of MG had significantly increased in all populations reported so far. Thus, recent Japanese study compared the age of onset and found that the mean age of onset changed from 35 years in 1982 to 1986, through 43 years from 1992–1996 to 49 years from 1997 to 2001 [52]. The same authors found significant increase of the MG age of onset among elderly population above 65 years of age, particularly among females [52]. In another study the mean age of onset is not mentioned, but the two peaks of high frequency of MG onset were reported for women aged 30–39 and 70–79, and for men just one peak appeared in the cohort aged 60–69 [79]. The data in the latest publication was obtained by age distribution of people with positive AchRab. At the same time, the increased rate of MG was reported for the disease form characterized by late-onset, while for early MG the rate was stable [85]. Interestingly, the age of disease onset seems to be dependent on genetic background, the early but not the late onset MG was associated with the HLA-DR3 phenotype [85].

The above nicely illustrates either the changing dynamics of the disease development with regard to patients' age (on the more general level stressing that when autoimmunity and autoimmune diseases are analyzed against the individuals age, nothing is certain and the paradigms can change, affecting also the clinical thinking), or the changing knowledge related to the diseases' identification, diagnosis, etc. Among the important factors that may contribute to these changes, may be recently increased interest of scientist in the health of elderly population, caused by significantly increased numbers of people aged more than 65 in most "western" countries and thus, more data available for this population. Another possibility is the true increase in rate of late-onset MG, both connected and not connected with thymoma, depending on reports [1, 110]. The authors who reported increased frequency of MG in elderly people are rather suggesting environmental factors as its cause, but since the phenomenon is reported in many distant regions of the world, including USA, Europe and Japan, the existence of the same environmental factor(s) seems unlikely. Increased rate of late onset MG could depend on changed immune system of the

elderly people. Some initial findings, including an observation that the serum level of anti-AChR antibodies is often lower in elderly onset of MG patients than in the young group, but antibodies to titin can be found in half of elderly, and these latter are uncommon in young patients [73, 83, 85]. Myasthenia patients with positive serum anti-titin antibodies were older than those without these antibodies, mean age 58 years old versus 36 years old, respectively [73]. In this recent study the additional subgroup of MG was defined based on appearance of anti-ryanodine receptor antibody, and clinical features—the highest rate of bulbar, respiratory and neck involvement at MG onset; the mean age of this group was about 57 years old [73].

This information could be an another illustration of the above-described hypothesis, that autoantibodies against intracellular antigens could have a different origin than those directed against the surface antigens, since the anti-titin and anti-ryanodine receptor antibodies were found preferably in elderly patients with myopathies (or muscle destruction; i.e., [74, 86] their appearance required first the damage of muscle fibers to release intracellular antigens).

Summarizing, the prevalence of some autoantibodies is increasing with advance age but the frequency of new incidences of autoimmune diseases is not following. One very good illustration of this statement is found in a paper, in which the frequencies of autoantibodies in the sera of Danish unselected centenarians are reported at 79%, but autoimmune diseases confirmed by medical records were present only in 20%; in addition, the majority of them were diagnosed with pernicious anemia (10%), 3% with rheumatoid arthritis, and another 3% with *Polymyalgia rheumatica*. Graves' disease and autoimmune thyroiditis were rare (1% each) of examined people [4].

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Autoimmunity—Aging Mouse Model for Autoimmune Diseases

Yoshio Hayashi and Naozumi Ishimaru

Contents

Abstract: Recent evidences suggest that the apoptotic pathway plays a central role in tolerazing T-cells to tissue-specific self-antigen, and may drive the age-related autoimmune phenomenon. Primary Sjögren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the exocrine glands, and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La. It can be considered that a defect in activation induced cell death (AICD) of effector T-cells may result in the progression of autoimmune exocrinopathy in SS. We found that aging-associated disturbances in T-cell homeostasis are accelerated in the animal model with SS, resulting in the development of extraglandular manifestation including autoimmune arthritis and interstitial pneumonia. We demonstrated that tissue-specific apoptosis may contribute to autoantigen cleavage, leading to the age-related acceleration of autoimmune exocrinopathy. The immune system undergoes profound changes with advancing age that are beginning to be understood and that need to be incorporated into the pathogenesis of SS. The studies reviewed the molecular mechanisms on aging-associated progression in animal model of autoimmune exocrinopathy.

Keywords: Aging • Apoptosis • T-cell tolerance • Autoantigen • Sjögren's syndrome

Y. Hayashi (✉) · N. Ishimaru
Department of Oral Molecular Pathology
Institute of Health Biosciences
The University of Tokushima Graduate School
3 Kuramotocho, Tokushima 770-8504, Japan
Tel.: +81 88 633 7327
Fax: +81 88 633 7327
E-mail: hayashi@dent.tokushima-u.ac.jp

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1 Introduction: Primary Sjögren's Syndrome

Primary Sjögren's syndrome (SS) is generally considered to be a T-cell-mediated autoimmune disorder characterized by lymphocytic infiltrates and destruction of the exocrine glands, and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La [7, 10, 28]. It is assumed that autoreactive T-cells bearing CD4 molecule may recognize unknown autoantigen triggering autoimmunity in the exocrine glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) [24]. A combination of immunologic, genetic and environmental factors may play a key role on development of autoimmune lesions in the exocrine glands [24]. Lymphocytes first surround the salivary ducts and then extend into the acinar epithelium, leading to diminished glandular secretion as a result of apoptosis. Indeed, epithelial cell activation has been proposed by some to be the major pathological process in SS with increased expression of MHC Class II antigens, and Fas on epithelial cells in this disease [35]. There is an infiltration of a minor proportion of B-cells besides T-cells into the exocrine glands of SS [8]. Infiltrating B-cells account for up to 20% of the cells found in the salivary tissue. It is reported that B-cell activation leads to the production of autoantibodies and polyclonal hypergammaglobulinaemia characteristic of SS and the B-cell activation may account for the increased propensity of these patients to developing lymphomas [29]. BAFF is a member of the TNF superfamily and is involved in B-cell maturation and survival. It is found at increased levels in serum, salivary tissue and synovial fluid of patients with SS, and is expressed by T-cells [25]. Moreover, BAFF transgenic mice display a phenotype similar to SS or SLE [11]. These data imply an important role for T-and B-cell interaction in the pathogenesis of SS. On the other hand, it is well-known that a wide spectrum of extraglandular manifestations involving skin, joints, lung, heart, kidneys, nervous system and hematological and lymphoproliferative disorders may occur in SS patients [43], but the mechanisms for in vivo progression in autoimmune condition are still obscure. Although an important role for T-cells on the development of autoimmune disease has been argued, it is not known whether disease is initiated by a restrained inflammatory reaction to an organ-specific autoantigen. It is possible that individual T-cells activated by an appropriate self-antigen can proliferate and form a restricted T-cell clone. Previously, we have identified a 120 kDa α-fodrin autoantigen in the pathogenesis of primary SS [12], but the role of autoantigen which render in vivo immunoregulation remain unclear.

2 Crucial Role of Apoptosis

Apoptosis plays an important role in maintaining T-cell repertoire and deletion of autoreactive T-cells [3, 44], and is regulated by a number of gene products that promote cell death or extend cell survival [14, 23]. Fas ligand (FasL) mediates cell death by cross-linking Fas receptor in apoptosis-sensitive Fas^+ cells [4, 29]. It is now evident

that the interaction of Fas with FasL regulates a large number of pathophysiological process of apoptosis including autoimmune diseases [5, 18, 26, 40]. Recent studies have now confirmed the observation that apoptotic cells in various cell types implicated as the sourse of autoantigen when stimulated with different proapoptotic stimuli [6, 31, 38]. Much evidence shows that β-cell apoptosis is a fundamental process involved in the pathogenesis of Type 1 diabetes [1, 27]. In addition to apoptosis being the main mechanism by which β-cells are destroyed, β-cell apoptosis has been implicated in the initiation of Type 1 diabetes mellitus. These studies support that exaggerated β-cell damage can induce activation of β-cell-specific T-cells. In addition, nonobese diabetic (NOD) mice exhibit a defect in the clearance of apoptotic β-cells [34]. Therefore, the apoptotic cells may be a critical determinant contributing to the initiation of autoimmunity by having the capacity to instruct antigen-presenting cells (APCs) to modulate immune responses so that the outcome is T-cell activation. Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases [5, 45], accumulated evidences suggest an important role of apoptosis in the disease pathogenesis of SS [15, 17, 32, 41].

3 Age-related Decline in T-cell Functions

It is well-known that aging is associated with immunological defects, especially at the level of T-cells [13, 30, 36]. The mechanisms governing hyporesponsiveness in aged T-cells are poorly understood. The repertoire of naive and memory T-cells is less diverse, possibly as a result of thymic insufficiency, and it is biased toward autoreactive cells. Aging is associated with progressive decline in T-cell functions, including decreased response to mitogens, soluble antigens, and production of IL-2, expression of IL-2R, decrease in naive and increase in memory cells, and defect in signaling pathway [33, 39]. Activation-induced cell death (AICD) is a well-known mechanism of peripheral T-cell tolerance that depends upon an interaction between Fas and Fas ligand (FasL) [4]. AICD plays a central role, especially in killing autoreactive T-cells and in preventing autoimmune responses [44]. It has been reported that activation of T-cell clones induces FasL expression and AICD in autoreactive T-cells in vivo has been proposed to limit the expansion of an immune response by eliminating effector cells [46]. Previous studies have demonstrated that $CD4^+$ T-cells are susceptible to AICD induced through T-cell receptor (TCR) mediated recognition of allogeneic MHC Class II molecules, supporting the notion that AICD can be triggered in activated T-cells through the TCR-mediated recognition of antigen [20, 37]. These observations have suggested that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of SS. We detected a significant increase of $TUNEL^+$apoptotic epithelial duct cells in the salivary glands in the aged SS model of NFS/*sld* mice than those in the young model (Fig. 1). We found that Fas expression on the cultured mouse salivary gland (MSG) cells from the aged SS model mice was significantly augmented than those in the young model. Indeed, severe destructive

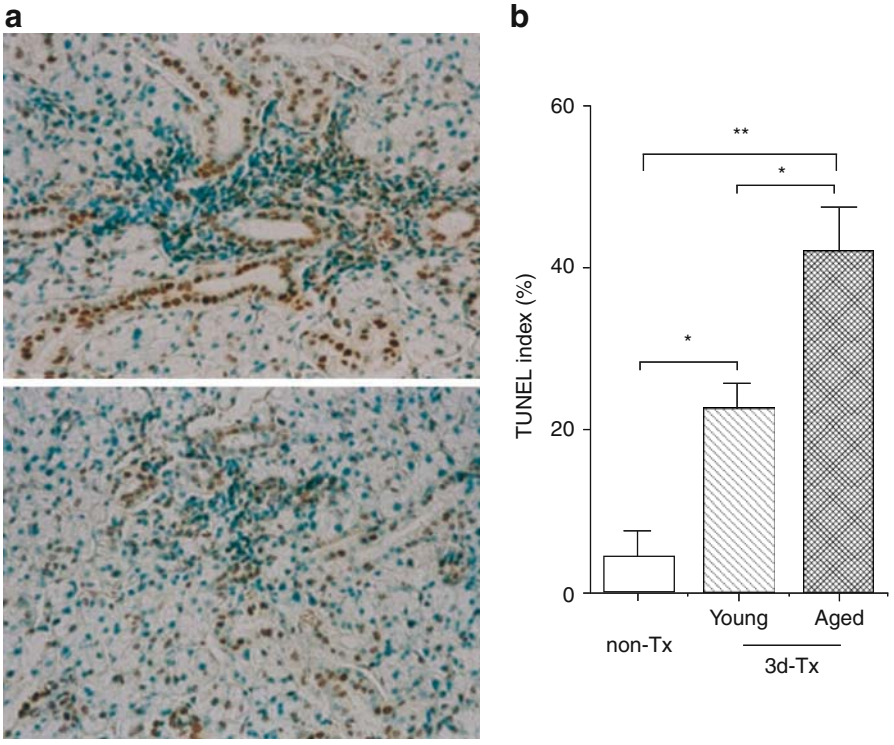

Fig. 1 Severe destructive autoimmune lesions in Sjögren's syndrome (SS) model mice with aging (Ref. 16). (a) Detection of TUNEL$^+$-apoptotic epithelial duct cells in the salivary gland tissues from aged SS model mice (18–20-mo-old, upper panel), and those in the young group (2– 4-mo-old, lower panel). (b) A significant increase of TUNEL$^+$-apoptotic epithelial duct cells was observed in the salivary gland tissues from aged SS model mice, compared with those in the young group. The percentage of duct cells staining positively with TUNEL was enumerated using a 10 x20 grid net micrometre disc, covering an objective of area 0.16 mm2. Data were analyzed in 10 fields per sections, and were expressed as mean percentage ± SD in 5 mice examined per each group (asterisks*, $p<0.01$ & asterisks**, $p<0.001$, Student's t-test)

autoimmune lesions in the salivary and lacrimal glands were observed in the aged SS animal model [16]. An increased expression of Fas by MSG cells has been shown to have a major influence on the susceptibility of severe tissue destruction in the aged salivary and lacrimal glands. These results indicate that the aging-associated acceleration of apoptotic cascade developed in the SS model mice.

4 Extraglandular Manifestation

A wide spectrum of extraglandular manifestations including arthritis may occur in SS patients [43]. Rheumatoid arthritis (RA) is a disease of adults with the highest incidence rates reported in the elderly [21, 42]. The immune system undergoes profound

changes with advancing age that are beginning to be understood and that need to be incorporated into the pathogenetic models of RA. Age-dependent disturbance in T-cell homeostasis are accelerated in patients with RA [2]. A defect in AICD of effector T-cells may result in the development of autoimmune disease [9], but an in vivo role of autoantigen for AICD with aging is entirely unclear. We examined the in vivo age-related changes on the development of extraglandular manifestations of autoimmune lesions in the NFS/*sld* SS model mice, compared with control young mice. Inflammatory lesions in aged SS model were observed in several organs including joints, lung, liver, and kidney, and most prominent histopathology was obtained in arthritic lesions. Destructive autoimmune arthritis developed in aging SS model mice, and these lesions aggravated with age [22]. The effects observed in aged SS model mice included synovial hyperplasia, pannus formation, bone erosion, and infiltration of mononuclear cells into the subsynovial tissue (Fig. 2). Culture supernatants from anti-CD3 mAb-stimulated splenic T-cells obtained from aged SS model mice contained higher levels of IL-2, and IFN-γ with advance of age, while no different levels of IL-4, and IL-10 were observed by ELISA. We detected increased levels of serum RF, anti-CII Abs (CII: type-II collagen, candidate autoantigen of RA), anti-ssDNA and anti-CII Abs in aging SS model mice but not in control mice, and these levels increased

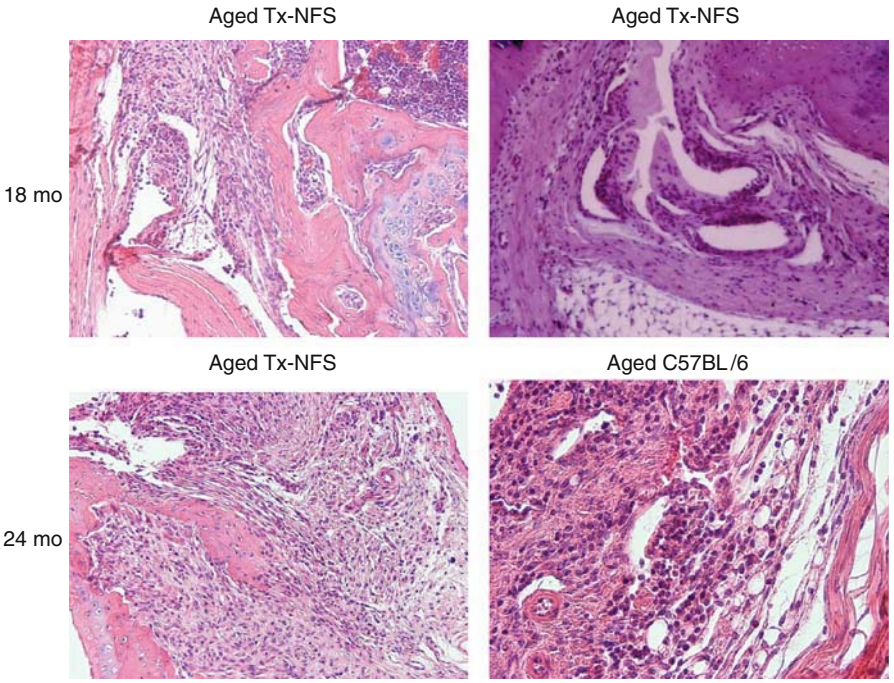

Fig. 2 Effects of aging on joint histopathology in SS model mouse (Ref. 22). Representative photomicrographs taken from SS model mice at 18-mo- and 24-mo-old. The histopathological effects observed in aged SS model mice at 18-mo- and 24-mo-old included pannus formation, synovial hyperplasia and infiltration of mononuclear cells into the subsynovial tissues (H.E.)

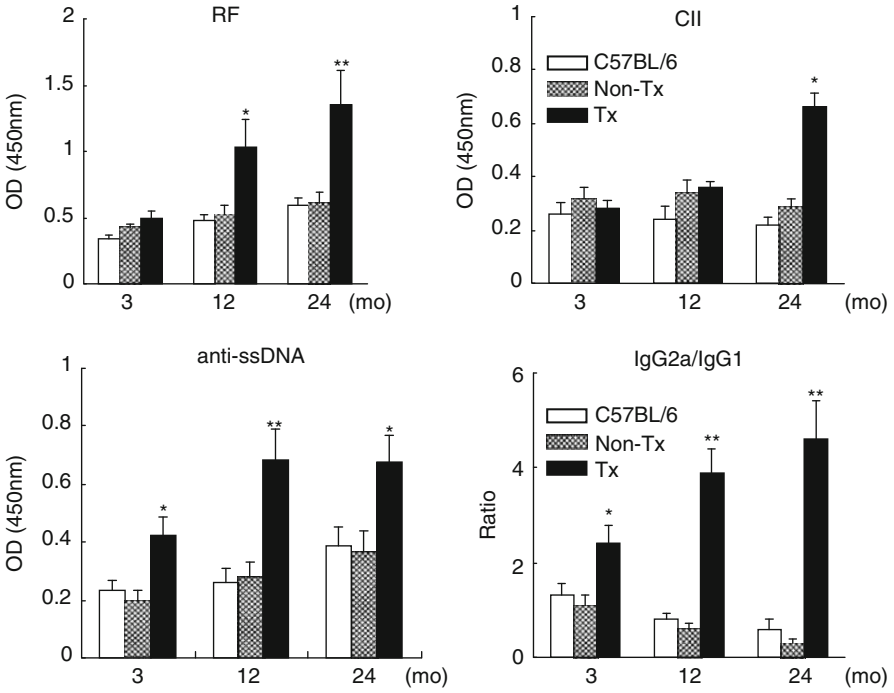

Fig. 3 Age-related changes in SS model with serum autoantibody productions (Ref. 22). Increased levels of serum RF were observed in aged SS model (*p<0.05 at 12-mo-old and **p<0.01 at 24-mo-old, Student's t test), compared with those in control mice. Significant increase in serum anti-CII was observed in aged SS model (*p<0.05 at 24-mo-old, Student's t test), compared with those in control mice. Contiguous increases in anti-ssDNA Abs in SS model mice were found at different age (*p<0.05 at 3-mo-and 24-mo-old and **p<0.01 at 12-mo-old, Student's t test), compared with those in control mice. High IgG2a/IgG1 ratio in sera from SS model mice was detected with advance of age, compared with those from control mice (*p<0.05 at 3-mo-old and **p<0.01 at 12-mo- and 24-mo-old, Student's t test)

with advance of age (Fig. 3). A high titer of serum autoantibodies against N-terminal α-fodrin fragments (JS-1) that originally identified in primary SS model mice were detected in the aged SS model mice by ELISA. Moreover, autoantibody production against C-termini of α-fodrin fragment (3 DA) was frequently detected in aged SS model mice. To address the role of autoantigen-reactive T-cells, we examined the proliferative T-cell responses against α-fodrin fragments (JS-1, 2.7A, and 3'DA), and CII in the spleen cells at different ages. We detected a significantly increased proliferation in spleen cells from aged SS model mice stimulated with CII, in addition to the response with JS-1, 2,7A and 3'DA protein (Fig. 4). By contrast, impaired proliferative responses were observed when stimulated with anti-CD3, and LPS with advance of age. These data suggest that α-fodrin-reactive T-cells may spread against different antigenic epitope, followed by bystander T-cell activation involving against CII on the progression of autoimmune lesions in aged SS model mice [22]. Furthermore,

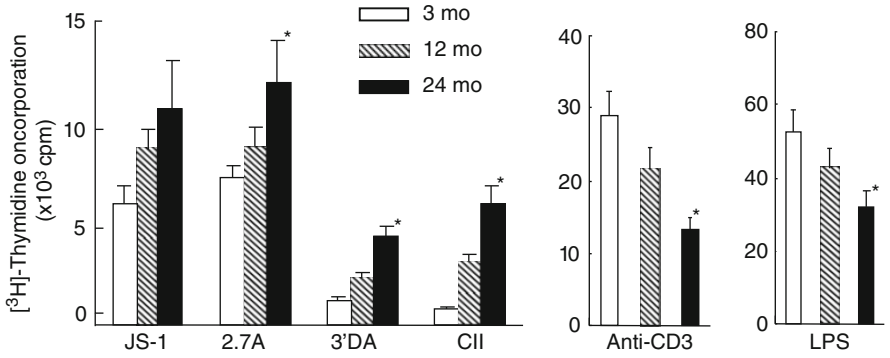

Fig. 4 Possible role of bystander T-cell activation on the development of autoimmune arthritis in aging SS model (Ref. 22). A significantly increased proliferation in spleen cells from aged SS model mice stimulated with JS-1,2,7A and 3'DA protein (asterisk*, $p<0.05$, Student's t test). Moreover, a significant increase in CII-specific T-cell proliferation was found in the aged SS model mice with advance of age (asterisk*, $p<0.05$, Student's t test). In contrast, decreased proliferative responses were observed when stimulated with anti-CD3, and LPS with advance of age (asterisk*, $p<0.05$, Student's t test). Data are expressed as counts per minute per culture ± SD in triplicate

interstitial pneumonia of autoimmune nature was involved in aging SS model mice until 24-months-old. The results showed that $CD4^+$ T-cell population, $CD4^+$ T-cells bearing $CD44^{high}$, Mel-14^{low}, $CD45RB^{low}$ activation markers, and MHC class II+ cells were significantly up-regulated in the spleens from SS model mice with aging. These results indicate that age-related disturbance of T-cell tolerance may play a crucial role on the involvement of interstitial pneumonia besides autoimmune arthritis in a murine SS model.

5 Concluding Remarks

The data discussed in this review are strongly suggestive of essential roles of apoptotic cascade for α-fodrin autoantigen cleavage leading to age-related acceleration in autoimmune exocrinopathy. In vitro T-cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from aging model for SS. Although antigen-induced T-cell death is known to be regulated by CD4 expression, molecular mechanisms responsible for T-cell death should be further elucidated. Moreover, it remains unclear whether T-cells specific for endogenous epitopes play a significant pathologic role in tissue damage during the clinical episodes. Taken together, aging-associated disturbance in T-cell homeostasis have a potent effect on the proteolysis of α-fodrin autoantigen through up-regulation of apoptotic activity. Increasing our knowledge of the biology from different aspects will be of vital importance for the future application of immune suppressive therapy of aging-associated autoimmunity.

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Atherosclerosis—An Age-dependent Autoimmune Disease

B. Henderson, A. Rossmann, Ch. Mayerl, M. Wick and G. Wick

Contents

1 Introduction

1.1 Age-dependent Diseases

With increasing age, various diseases that mostly have their roots earlier in life become clinically manifest and are, therefore, age-dependent diseases. Table 1 lists the most frequent medically as well as the most important diseases from a socio-economical perspective. However, several age-related diseases often develop in a single patient and this multimorbidity is the major problem in geriatrics that

G. Wick (✉) · B. Henderson · A. Rossmann · Ch. Mayerl
Division of Experimental Pathophysiology and Immunology
Laboratory of Autoimmunity, Biocenter
Innsbruck Medical University
Fritz-Pregl-Str. 3/IV
A-6020 Innsbruck, Austria
Tel: +43/512/9003/70960; Fax: +43/512/9003/73960
E-mail: Georg.Wick@i-med.ac.at

M.Wick
Department of Radiology
Innsbruck Medical University
Anichstrasse 35, A-6020 Innsbruck, Austria

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Table 1 Major Age Associated Diseases

• Cardiovascular disease (MI, Stroke, Claudication)
• Diabetes, Obesity, Metabolic-Syndrome
• Tumors (Breast, Lung, Prostate, Colon)
• Osteoporosis
• Arthrosis
• Dementia (Vascular, Alzheimers Disease)
• Depression

becomes ever more important with increasing life expectancy. Worldwide, cardiovascular diseases and infections are the main causes of death followed by tumours [1]. Interestingly, in the developed world, the costs imposed upon society by treatment of age-related diseases shows a different distribution, i.e., cardiovascular diseases followed by autoimmune diseases and tumours with infections at lower ranks. Among cardiovascular diseases, atherosclerosis is the most important representative leading to the known severe sequelae, i.e., myocardial infarction, stroke and peripheral arterial occlusion. In recent years, increasing experimental and clinical evidence has emerged supporting the concept that inflammatory-immunological processes play a major role in the initiation and progression of atherosclerosis including the hypothesis of a triggering of atherogenesis by microbial-human antigenic crossreactivity as well as *bona fide* autoimmunity.

The following chapter will focus on this latter aspect.

1.2 Pleiotropic Antagonism

In principle, theories of aging comprise two major groups viz.:

(a) aging due to stochastic processes (random damage to DNA, RNA, proteins)
(b) deterministic concepts (genetically determined lifespans, biological clocks, etc.).

Our own gerontological research with respect to the elucidation of the pathogenesis of age-dependent diseases was always based on the concept of pleiotropic antagonism (pleiotropic = *Gr*, multifunctional; antagonistic = *Gr* having the opposite effect). In the present context, pleiotropic antagonism means that genes, the effect of which is beneficial during youth, may exert deleterious effects at older age when natural selection is not active anymore [2]. These deleterious (i.e., antagonistic as compared to beneficial expression before reproductive age) effects are pleiotropic (i.e., occur at different sites and tissues). Examples for pleiotropic antagonism as an explanation for the development of age-dependent diseases have been discussed in detail earlier [3, 4]. They include the activation of genes responsible for the biochemical processes leading to the calcification of bones early in life that can later be expressed in the arterial wall, leading to the development of severe atherosclerotic lesions. Another example is the benign prostatic hypertrophy (BPH) that in part is due to the autocrine and paracrine activity of growth factors contained in prostatic fluid. In youth, these factors are beneficial for the survival of spermatozoa and proliferation of prostatic parenchymal cells with the aim to produce large amounts of

seminal fluid that will facilitate reproductive success. However, in older age when sexual activity decreases, these factors together with a decrease of proapoptotic components that are also contained in seminal fluid will contribute to the development of BPH.

In conclusion, age-dependent diseases are often the "price that we pay for the vigour of youth".

2 Hypotheses for the Development of Atherosclerosis

The term *atherosclerosis* refers to an arterial lesion containing foam cells and interstitial lipid deposition. In contrast, *arteriosclerosis* is a collective term, summarising different metabolic and degenerative arterial alterations that cause hardening (sclerosis), thickening, and loss of elasticity of the arterial wall. Atherosclerosis encompasses spontaneous atherosclerosis [5], restenosis after percutaneous transluminal coronary angioplasty, autologous arterial or vein graft atherosclerosis and transplant atherosclerosis, the latter an accelerated form of atherosclerosis. The atherosclerotic lesion is defined by arterial intimal and smooth muscle cell (SMC) proliferation, lipid accumulation, and connective tissue deposition [6, 7]. Arteriosclerosis is characterised by SMC hyperplasia or hypertrophy and extracelluar matrix (ECM) protein accumulation in the intima and/or media with or without lipid deposition, resulting in thickening and stiffness of the arterial wall. A common feature of all these vascular diseases is related to altered hemodynamic stress [8]. Although arteriosclerosis is the umbrella term of all these forms of vascular diseases including atherosclerosis, the 2 terms are often used interchangeably and we will thus refer to atherosclerosis throughout this article.

2.1 Classical Hypotheses of Atherosclerosis

The main classical hypotheses for atherogenesis are the following:

(a) *The response to injury hypothesis [9]*
This concept assumes a primary endothelial cell damage as the initiating event of the disease. This injury can be brought about by mechanical stress (hypertension), oxygen radicals, toxins (e.g., from cigarette smoke), etc. and entails an increase in vascular permeability and the expression of growth factors, proinflammatory cytokines and adhesion molecules. Although lymphoid cells were already recognised in the intima at the time when this theory was formulated, no major early pathogenetic relevance was initially attributed to this finding.

(b) *The arterio-ELAM theory [10]*
This is a supplementary concept to the response to injury hypothesis, where endothelial dysfunction is also considered a primary event in the pathogenesis of atherosclerosis, followed by infiltration of monocytes/macrophages into the intima where they transform into foam cells. These cells produce proinflamma-

tory cytokines, such as IL-1 and TNFα, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) that together with infiltrating T-cells stimulate the proliferation of SMC and endothelial cells. These cellular changes entail the expression of inducible endothelial leukocyte adhesion molecule (ELAM) and other adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM 1), and form the basis for a perpetuation of the atherogenic process.

(c) *The altered lipoprotein hypothesis [11]*
This hypothesis states that chemically altered, e.g., oxidized, low-density lipoproteins (oxLDL) represent the trigger for the development of atherosclerotic lesions. OxLDL is deposited or formed *de novo* in the arterial intima where it acts as a chemoattractant for monocytes from the blood stream on one hand, and is taken up by macrophages and SMC in the intima via nonsaturable scavengor receptors on the other. The latter process leads to the formation of foam cells and extracellular cholesterol deposits resulting in the development of so called fatty streaks that for a long time where considered as being the first atherosclerotic manifestations.

(d) *The autoimmune hypothesis [12]*
This newer hypothesis formulated by our group that is described in detail below seems to encompass all the former classical theories described so far. In this context it is important to emphasize that our laboratory is only interested in the very earliest events leading to clinically not yet apparent atherosclerotic changes that may later progress into more severe lesions if classic atherosclerosis risk factors are continuously present.

2.2 Inflammation and Atherosclerosis

The fact that inflammatory processes could be observed in atherosclerotic lesions was already pointed out by several authors in the 19th century [13]. However, this observation has fallen into oblivion until it was rediscovered in the early 1980s and condensed into new, inflammatory hypotheses of atherogenesis, albeit without a clear cut knowledge if the inflammatory events were of a primary or secondary nature [14]. As a matter of fact, in the middle of the 19th century, the 2 leading European pathologists, Rudolf Virchow, Berlin, Germany, and Carl von Rokitansky, Vienna, Austria, were engaged in a fierce dispute about this issue. Both of them observed inflammatory mononuclear cell infiltration in atherosclerotic lesions, but while von Rokitansky considered these as of secondary in nature, Virchow assigned a primary pathogenetic role to them. We recently had the opportunity to reevaluate atherosclerotic specimens harvested by von Rokitansky himself over 150 years ago and stored at the pathology museum at the General Hospital in Vienna with modern immunohistochemical methods. Our data support the position of Virchow rather than that of von Rokitansky [15].

It later became increasingly clear that in advanced atherosclerotic lesions, i.e., those that are also available as surgical specimens for detailed studies, a great number of facets of inflammatory-immunological hallmarks can be found, thus rightly supporting the designation of these as “complicated lesions”.

In principle, hallmarks of both innate and adaptive immune reactions have been described in atherosclerosis. With respect to humoral factors these include depositions of complement, C-reactive protein (CRP) and a great number of cytokines and chemokines. Intralesional cellular components of the innate immune system comprise macrophages and NK T-cells, but not granulocytes. In this context, the expression of TOLL-like receptors (TLRs), both on effector and target cells, e.g., endothelial cells (ECs), is of special relevance [16].

With respect to the adaptive immune system, T-cells dominate the scene with a preponderance of $CD4^+$ over $CD8^+$ cells. Within the $CD4^+$ population, Th1 cells predominate over Th2 cells. Most T-cells express the α/β T-cell receptor (TCR α/β) but a surprisingly high percentage of intralesional T-cells is TCRγ/δ^+, i.e. 10–15% versus 1–2% in the peripheral blood of the same individuals [17]. This fact later became important because it is known that TCRγ/δ^+ T-cells recognize heat shock proteins (HSPs) in a non-MHC restricted fashion. In immunohistological studies comparing early lesions obtained from young (<30 yrs) donors who died from non-CVD-related causes with late lesions from older (>60 yrs) patients with severe plaques, it became clear that the first cells to infiltrate the intima at the known predilection sites are T-cells only then followed by blood-borne macrophages and finally smooth muscle cells (SMC) migrating from the media [18]. In addition, both early and late lesions contain abundant numbers of dendritic cells (DC) providing the conditions for local antigen presentation or transport of antigenic material to draining lymphnodes for antigen-presentation, and subsequent migration of sensitized T-cells to lesion areas, similar to the situation in contact dermatitis. Finally, early lesions contain considerable numbers of mast cells that secrete vasoactive products which increase vascular permeability [19].

Immigration of these different cell types is guided by appropriate chemokine gradients and the expression of chemokine surface receptors. Before intimal infiltration, mononuclear cells interact with ECs via adhesion molecules, the expression of which differs at predilection sites compared to the rest of the arterial territories. While ECs at the latter locations are subjected to laminar shear stress, the former sites experience turbulent blood flow conditions that build up after vascular branching, and lead to the expression of ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), and ELAM-1 (endothelial leukocyte adhesion molecule-1) all aiding the adhesion of cells.

These adhesion molecules are excessively expressed by ECs subjected to classical atherosclerosis risk-factors and—as will be detailed later—are a prerequisite for the interaction of T-cells with specific antigens expressed on the surface of target ECs. The binding affinity of the TCR for an appropriate MHC-peptide complex is only about 10^{-5} molar i.e., does not suffice for the interaction of effector and target cells in the arterial blood stream. However, after firm adhesion of T-cells onto ECs expressing appropriate adhesion molecules, specific immunologic interaction becomes possible. Similar rules apply for the interaction of monocytes with ECs with subsequent infiltration of the intima and transformation into macrophages.

In conclusion, all these findings supported a role of innate and adaptive immune reactions during atherogenesis, but the relevant antigen(s) triggering such reactions were not identified.

2.3 The Autoimmune Hypothesis of Atherosclerosis

In order to identify one or several (auto) antigens that may induce an immune response in the arterial wall, we resorted to the original approach of Rose and Witebsky for the identification of disease-specific autoantigens [20]. We immunized normocholesterolemic rabbits with a mixture of proteins isolated either from dilapidated human atherosclerotic plaques or from plaques of atherosclerosis-prone rabbits (so-called Watanabe rabbits) that have a genetic defect of the low density lipoprotein receptor (LDL-R), mixed with complete Freund's adjuvant (CFA). Ovalbumin (OVA) plus CFA was used for control purposes. We hypothesised that rabbits immunized with either human or rabbit plaque proteins should develop atherosclerotic lesions if atherogenic autoantigens were present in these preparations, while OVA immunized rabbits should remain unaffected. To our surprise, all three groups developed mononuclear cells infiltration at the known atherosclerosis predilection sites [21]. Since CFA was the common denominator, we then immunized rabbits with CFA alone and used incomplete Freund's adjuvant (IFA) for controls. In these experiments, CFA immunized animals again developed atherosclerosis while IFA immunized animals remained normal. Immunization with other nonmycobacterial containing adjuvants (e.g., lipopeptide) also had no atherogenic effect. CFA consists of heat-killed mycobacteria, mineral oil and the emulsifier Arlacel. An active component of mycobacteria is heat shock protein 65 (mHSP65) and we therefore continued these series of experiments by immunizing rabbits with recombinant mHSP65, which again led to the development of atherosclerosis.

Lesion-derived T-cell preparations showed a significantly higher reaction with mHSP65 than T-cells isolated from the peripheral blood of the same animals [22]. If mHSP65 immunized rabbits were additionally fed a cholesterol-rich diet, much more severe lesions developed [23]. While lesions in mHSP65 only immunized rabbits were still reversible, the more severe changes in mHSP65 immunized hypercholesterolemic animals were not reversible, at least during an observation period of 32 weeks.

From these and other data, we developed a new "Autoimmune Hypothesis for Atherogenesis" identifying HSP60 as a culprit antigen initiating the first inflammatory, clinically still inapparent stage of the disease that subsequently develops into more severe forms when classical atherosclerosis risk-factors persist [24–26]. The basis for this hypothesis is the fact that HSP60 is a phylogenetically old and highly conserved protein [27]. Thus, HSP60 from different bacterial species display over 97% homology and bacterial and human HSP60 still show about 55% homology, exceeding 70% at certain molecular domains. In addition, microbial HSP60 is not only a quantitatively but also qualitatively important constituent that exhibits strong immunogenicity. Every healthy human and animal has humoral and cellular immunity against microbial HSP60, as well as *bona fide* physiological autoimmunity against biochemically altered antologous HSP60 that is released during cellular necrosis and has to be removed. Under normal circumstances, this anti-HSP60 immunity exerts positive protective effects and contributes to the survival fitness of the respective organism. However—as will be reiterated below—subjecting vascular

ECs to classical atherosclerosis risk-factors leads to the simultaneous expression of certain adhesion molecules and human HSP60 [28–30], the latter being recognized as a danger signal by the innate and adaptive immune system leading to an immunologic attack on these target ECs. Arterial ECs that are subjected to lifelong higher blood pressure than venous ECs have a significantly lower threshold for the adhesion molecule and HSP60-inducing effect of various stress factors such as classical atherosclerosis risk-factors. This is the reason why we develop arterio- rather than venosclerosis.

We do not, of course, deny the well-proven atherosclerosis-promoting effect of classical atherosclerosis risk-factors but we assign a different role to them in the very earliest stages of the disease, viz. acting as endothelial stressors. In the rare person that is exposed to various risk-factors but does not develop atherosclerosis, the atherogenic T-cell peptides of HSP60 apparently are not accommodated in the MHC class I or class II groves, a constellation on which one should, however, not count.

In conclusion, the early inflammatory stage of atherosclerosis is the price that we pay for the preexistent antimicrobial immunity and *bona fide* autoimmunity against HSP60 when we maltreat our vascular system with HSP60-inducing atherosclerosis risk-factors, such as high blood pressure, smoking, oxygen radicals including oxidized LDL (oxLDL), high blood cholesterol levels, diabetes, etc.

3 Heat Shock Proteins

The HSPs are a diverse family of proteins that perform vital roles in the cell, the most important of these being the folding and re-folding of proteins to their native structures, as well as intracellular protein/peptide transport (Table 2). Although the discovery of HSP was made in *Drosophila*, it soon became apparent that they were present in all organisms, whether eukaryotic or prokaryotic. Despite the evolutionary distance between these domains, sequencing various genes and proteins revealed the above mentioned highly phylogenetically conserved sequences, emphasising the functional importance of HSPs.

Table 2 The Heat Shock Protein Family

HSP Family	Molecular Function
10 kDa	Protein folding, co-chaperone with Hsp60
27 kDa	Inhibits protein aggregation, stabilises cellular structure
40 kDa	Protein folding, co-chaperone with Hsp70
60 kDa	Protein folding and re-folding, protein translocation
70 kDa	Nascent protein folding, refolding of denatured proteins, regulation of heat shock response, cell cycle and cell signalling, anti-apoptotic function
90 kDa	Protein folding, interaction with steroid receptors, controls HSF-1 activity by direct binding
100 kDa	Protein folding and re-folding after aggregation

Adapted from Jolly C, Morimoto RI. Journal of the National Cancer Institute 2000 92(19):1564-1572

The HSPs are ordered into families depending on their molecular weight, which varies from over 100 kDa to under 10 kDa. The major HSP families include the 100, 90, 70, 60, 40, and 28 kDa families, all of which perform various roles in protein folding and transport, and prevention or dissolution of protein aggregates [31, 32]. A special case here is HSP90, which not only binds to denatured polypeptides, but also performs many other tasks in cell cycle control, signal transduction, and even steroid receptor function. HSP expression is induced in cells that have undergone exposure to some form of stress, leading to an increase in the levels of unfolded or erroneously folded proteins. This occurs not only upon exposure to high temperatures (the stress that lead to the discovery of HSP), but also to a variety of other stressful situations, including heavy metals, UV light, reactive oxygen species, and bacterial or viral infection [31]. Induction of HSP occurs when the transcription factor, heat shock factor 1 (HSF-1), is released from its quiescent state by HSP70, which is sequestered to refold denatured proteins [33]. HSF-1 is now able to relocate to the nucleus and form a homotrimer, which is subsequently phosphorylated and binds to heat shock responsive elements (HSRE) which are present in the promoter regions of HSP genes. A negative feedback loop inactivates the HSF trimer as the excess HSP70 eventually binds to HSF and dissociates the complex.

Because of their ubiquitous nature, HSP have also been found to be implicated in a number of diseases, including arthritis, multiple sclerosis, and diabetes [34–38]. The role of HSP60 in atherosclerosis has already been mentioned, and will be discussed in further detail below.

4 Classical Atherosclerosis Risk-factors as Endothelial Stressors

The risk factors for atherosclerosis have been well defined from a number of large epidemiological studies over the last several decades, usually having myocardial infarction or ischemic stroke as an endpoint [39-43]. Some of these factors are (currently) unable to be addressed, such as being male, and the rest of the genetic makeup which ascribes a 3-fold higher risk to people who have two or more close relatives succumbing to atherosclerosis. The other risk factors are of an environmental nature, and can in principle all be described as capable of inducing a stress response in ECs.

High blood pressure is one of the most important risk factors in atherogenesis, and is aggressively treated in patients. As mentioned, it is well known that atherosclerotic lesions form at certain predilection sites in arteries, where a turbulent, non uniform, non linear blood flow is found. These sites are commonly found at branch points and even curved regions of arteries. Linear blood flow forms the normal environment for EC, and areas of disturbed flow cause the expression of a totally different set of genes [44], and increase the ability of mononuclear cells to attach and migrate across the endothelium. In addition, increased and turbulent mechanical stress has been shown to increase the expression of HSP60 on the protein level both in vivo and in vitro. In rats, ligation of one carotid artery dramatically increases blood flow in the other, after which HSP60 induction was shown both on a transcriptional and translational level [30]. Fur-

thermore, shear stress experiments on cultured endothelial cells using a cone and plate viscometer showed that turbulent flow induced HSP60 protein expression [30].

Numerous studies have found a strong link between smoking and an increased incidence of heart attacks and strokes [45, 46]. Indeed, smoking has even been found to be the major risk factor in the development of early ateriosclerosis [47]. In our laboratory, young male smokers aged 17–18 years were shown to carry a 3.5-fold higher risk of having an increased intima-media thickness (IMT) at one or more sites investigated by ultrasound compared to nonsmokers. We have investigated the effects of treating cultured EC with an aqueous cigarette smoke extract (CSE), which has been reported to contain the substances responsible for atherogenesis. We have shown in vitro and ex vivo that ECs undergo a strong cytoskeletal contraction due to the rapid degradation of microtubulin, which in vivowould lead to vessel denudation [48]. Microarray experiments have shown that CSE induces HSP60 expression, along with a large number of other heat shock and stress response genes (Henderson et al. in press; doi:10.1016/j.atherosclerosis.2008.02.022). HSP60 has also been shown to be released in copious amounts into the cell culture medium in response to CSE [49].

The role that LDL plays in atherosclerosis is also well known, with LDL permeating the endothelial monolayer to the intima, undergoing oxidisation there, and then being taken up by nonsaturatable scavenger receptors present on monocytes and smooth muscle cells to form foam cells, leading to the appearance of the classical fatty streak [50, 51]. Oxidised LDL is a strong EC stressor, with the effects on ECs of arterial origin stronger than that of venous origin [29]. This supports the assertion mentioned above that arterial EC are more susceptible to stressors due to the prestress by higher arterial blood pressure.

In an analogous fashion, proinflammatory cytokines represented by TNF-α, reactive oxygen species (H_2O_2), and bacterial infection (LPS treatment) have all been shown to induce HSP60 protein and adhesion molecule expression in EC in vitro [29]. Furthermore, bacterial LPS administration in rats led to simultaneously increased expression of HSP60 and ICAM-1 [52].

In recent, still unpublished experiments, we have visualized HSP60 expression by in vivo imaging techniques in rabbits that were i.v. injected with LPS as a surrogate for infections (M. Wick et al Cell Stress Chaperones. 2008 Sep;13(3):275-85). Fig. 1 shows this fact by *en face* immunohistochemistry.

In view of the ongoing "electrosmog" discussion, we have also investigated whether the 50 Hz magnetic fields produced by domestic power supplies could represent an endothelial stressor by assessing HSP60 and 70 expression in vitro and in vivo. After exposing EC to various intensities and durations of 50 Hz fields, no response of HSP60 or 70 was found on the RNA or protein level [53]. Further investigations using microarray analysis also failed to produce any candidate genes that were reproducibly affected by 50 Hz exposure [54]. To look at this issue in a more complicated system in vivo, a mouse model of arterio-venous bypass restenosis was exposed to 50 Hz magnetic fields over several weeks. No change in progression or in the cellular composition of restenosed bypass conduits could be found compared to control groups. In addition, no change in HSP60 expression could be determined, thus rendering a proatherogenic role of low frequency magnetic fields rather improbable [55].

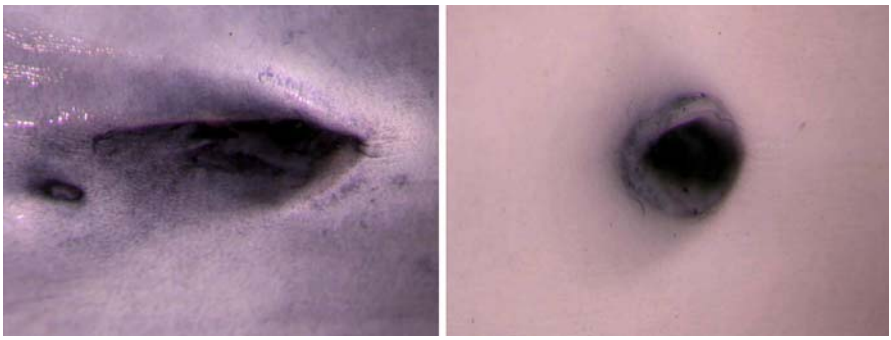

Fig. 1 *En face in vitro* immunohistochemical demonstration of HSP60 expression of a rabbit aorta branching into an intercostal artery using a mouse IgG2a anti-HSP60 monoclonal antibody (clone II-13) 24 hours after 10 μg/kg bodyweight LPS injection (left image), compared to a negative staining control using unspecific IgG2a (right image). Magnification:1.6x2.0

5 Role of the Adaptive Immune System

5.1 Humoral Immunity

Although B-cells are generally not found in atherosclerotic plaques in humans or mice [56], antibodies do play a role in atherosclerosis [18]. Fifteen years ago, we were able to demonstrate that sonographically visible carotid atherosclerosis significantly correlated with the antibody titer to mHSP65 [57]. It was subsequently shown that a positive correlation exists between antibody titer not only with morbidity and even with cardiovascular disease mortality [26]. Antibodies to mHSP65 have also been shown to cross-react with HSP from other pathogens (*Chlamydiae*, *E. coli*), and—importantly—with human HSP60 (hHSP60) [58, 59]. Linear and conformational cross reactive HSP60/65 have also been identified which form a starting point for further investigation of the role of antibodies in atherogenesis [60, 61]. A number of studies have since reproduced this data (reviewed in [12]), while other studies have found a role for antibodies against heat shock protein 70 [62]. However, immunisation with HSP70 fails to induce atherosclerosis, in contrast to mHSP65 (Wick et al. *unpublished data*). High levels of soluble HSP60 also correlate with an increased carotid IMT, which is exacerbated by the presence of chronic infections [63]. Interestingly, a negative correlation between disease and soluble HSP70 or HSP27 proteins has been shown [64, 65].

Other antigens have also shown a correlation of antibody titer and atherosclerosis. These include ox-LDL, where increased antibody concentrations have been linked to peripheral vascular disease and carotid and coronary atherosclerosis [66, 67]. As with HSP60 antibodies, increased ox-LDL antibody titres were correlated with disease progression and death [68, 69]. On the other hand, it has been shown that antiox-LDL antibodies are protective in mice, with their induction reducing the size of atherosclerotic lesions, probably due to the removal of ox-LDL from the blood

stream [70]. Anticardiolipin antibodies, which target b2-glycoprotein-I which is found in atherosclerotic lesions [71], were found to be elevated in patients with myocardial infarction and cardiac death [72].

5.2 Cellular Immunity

T-cells play an important role in atherogenesis and development of the atherosclerotic lesion (reviewed in [56]). After initial activation of the T-cell, generally in lymph nodes by antigens presented by dendritic cells, they are free to migrate into other tissues and become reactivated. Definitive evidence that CD4+ T-cells are atherogenic has been provided using mouse models of atherosclerosis. Immune compromised SCID mice show reduced atherosclerosis compared to immune competent mice, and reconstitution with CD4+ T-cells leads to more severe atherosclerosis [73]. The absence of CD4+ T-cells, or their depletion using anti-CD4 antibody were found to reduce fatty streak formation in C57BL/6 mice on a high fat diet [74]. Depletion of T-cells using an anti-CD3 antibody also lead to a reduction in atherosclerosis in normocholesterolemic rabbits which had been immunised with mycobacterial HSP65 [75, 76]. Proinflammatory cytokines produced by Th1 T-cells in the atherosclerotic lesion activate bystander cells, including macrophages and NK-cells, and are mainly responsible for the atherogenic effect of T-cells. The secretion of IFN-γ, IL-12 and IL-18, along with TNF family cytokines leads to the development of a proinflammatory environment. The role of these cytokines in atherosclerosis has been shown by various knockout mice lacking either the cytokines or their receptors [77–81]. Proinflammatory cytokine secretion by T-cells and activated bystander cells also leads to the expression of adhesion molecules, proteases, and ox-LDL scavenger receptor in various cell types, including EC and smooth muscle cells.

As previously mentioned, $CD4^+$ dominate over $CD8^+$ T-cells within lesions, with the vast majority of the former being α/b receptor positive. The fact that a considerably increased proportion of $CD4^+$ cells expressing the γ/δ receptor are also found provides an interesting link to innate immunity. Since γ/δ T-cells are capable of recognising antigens such as HSPs, without the need for MHC presentation, this introduces a link between the adaptive and innate immune systems [82]. The higher incidence of γ/δ T/cells in early atherosclerotic lesions is further evidence of the role of HSP60 in atherogenesis. Interestingly, knocking out TCRαβ T-cells on an ApoE-/- background led to a significant reduction in both early and late atherosclerosis, while deletion of the γδ T-cells had only a minor effect [83]. Antigen presenting cells (APC) such as dendritic cells and macrophages (expressing MHC II) are closely associated with T-cells in plaques, suggesting an interaction in situ [84]. It has been shown that T-cells undergo clonal expansion in atherosclerotic lesions. Using the Immunoscope technique, which is PCR-based and shows the clonal distribution of the Vβ T-cell receptor, a restricted T-cell repertoire has been found in late atherosclerotic lesions in humans [85, 86]. We were able to show that T-cells isolated directly from lesions

showed a significantly higher degree of monoclonally and oligoclonally restricted α/β T-cell receptor repertoire than those isolated from peripheral blood ($p<0.004$ and $p<0.003$ respectively; [87]). This suggests that plaque T-cells are proliferating in response to a specific antigen(s). The identity of these atherogenic epitopes has not been proven, but studies have shown that T-cells isolated from late lesions are reactive to HSP60 [87–90], and ox-LDL [91]. Other potential antigens include Chlamydial antigens, herpes simplex, cytomegalovirus, and β2-glycoprotein I which are all found in atherosclerotic plaques [71, 92].

Natural killer (NK) lymphocytes, which play an important role in the innate early defense response, have also been shown to be present in atherosclerotic lesions, albeit at very low levels, independent of lesion progression [93]. The deficiency of NK-cells in LDL receptor knockout mice led to a significant reduction in early lesion development, although its role in atherosclerosis is yet to be defined [94].

NK T-cells (NKT) are a heterogenous group of cells which possess characteristics of both NK- and T-cells, and act as a link between the innate and adaptive immune responses. Activation of NKT by IL-12 leads to the production of TNF-α and IFN-γ enabling the stimulation of macrophages, NK-cells and T-cells. NKT also express proinflammatory cytokines IL-2 and IL-12, and have also been shown to express Th2 cytokines IL-4, IL-5, and IL-10 [95]. Several publications have shown that NKT-cells contribute indirectly to the development of atherosclerotic lesions in mouse models, via activation of other immune cells and the production of a proinflammatory local environment [95–97].

Dendritic cells play an important role in the stimulation of T-cells and NKT-cells. Indeed, they are the most effective antigen presenting cells, capable of displaying antigens via MHC class I and II molecules, and controlling the differentiation of T-cells into Type 1 or Type 2 effector cells depending on IL-12 secretion [98–102]. The presence of co-stimulatory molecules (CD40, CD80/B7.1, CD86/B7.2) on the surface of DC are critical in the activation of T-cells, while their absence will cause T-cell anergy or even apoptosis [98–102]. DC were first reported to be present in severely diseased arteries by Bobryshev and Lord in 1995 [84], and later shown to be present in high numbers in healthy arteries but not veins, where they make up part of what we have termed the vascular associated lymphoid tissue (VALT) [103]. The VALT is partly analogous to the mucosa associated lymphoid tissue (MALT), and consists of mononuclear cells in the intima (T-cells, dendritic cells, mast cells, and macrophages) at known predilection sites for atherosclerotic lesions [93]. Present even in the arteries of babies and young children, the VALT is thought to have a similar role to the MALT: i.e., being a local immune monitoring site for potentially dangerous autologous and exogenous antigens present in the blood, an internal surface barrier region. DC present in atherosclerotic lesions have been shown to be far more abundant than in nondiseased arteries, and to be activated (shown by increased expression of adhesion molecules Cd11a, CD50, CD54; costimulatory factors CD58, $CD80^+$, $CD86^+$; and antigen presenting Class I and II MHC molecules and CD1) in contrast to normal tissue [104, 105]. This implies that DC undergoes maturation during atherogenesis, although the exact timing and nature remain unknown. Clusters of DC are essential to, and have been shown to be an indicator

of autoimmune disease also supporting the HSP60 autoimmune hypothesis outlined earlier [106].

5.3 Immune Regulation

Regulatory T-cells (Tregs) are a subset of $CD4^+$ or $CD8^+$ T-cells that exert an important regulatory effect on the cellular immune system by secreting cytokines (principally IL10 and TGF-β) suppressing T-cell proliferation in response to antigenic activation. $CD4^+$ Tregs occur naturally and are produced in the thymus expressing CD25 and the transcription factor Foxp3, and may also be induced in the periphery from Tr1 T-cells as a response to antigenic stimulation and do not express Foxp3 [107, 108].

A lack of $CD4^+/CD25^+$ regulatory T-cells, has a profound effect on the extent of atherosclerosis in mouse models. Since CD25-/- mice quickly succumb to autoimmune diseases, as do irradiated ApoE mice reconstituted with CD25-/- bone-marrow or T-cells, an alternative approach was required. This was elegantly solved by looking at CD80/CD86 and CD28 deficient mice, which are no longer able to form the CD80/86-CD28 interaction necessary for Treg generation [109]. Mice lacking either CD80/86 or CD28 suffered from atherosclerotic lesions twice the size of wildtype controls. This effect was shown to be due to increased $CD4^+$ T-cell suppression by IL-10 and TGF-β [109].

In addition, the number and potency of Tregs was found to be reduced in the ApoE-/- mouse model of hypercholesteremia compared to C57BL/6 wild-type littermates [110]. Furthermore, 6 month old mice had significantly fewer Treg than 6 week old mice without atherosclerotic lesions. The transfer of $CD4^+/CD25^+$ cells into ApoE-/- mice led to a reduction in the size of atherosclerotic lesions associated with an increased aortic expression of IL-10, while $CD4^+/CD25^-$ cells led to more vulnerable plaques [110]. The same authors had previously shown an association between acute coronary syndromes and a deficiency in Treg numbers and activity in humans [111].

The generation of HSP60 specific $CD4^+CD25^{high}$ regulatory T-cells has recently been shown to dramatically decrease lesion size in ApoE-/- mice fed a high fat diet [112]. Using immature DC that had been loaded with HSP60, specific Treg cells were generated in vitrobefore being injected into ApoE knockout mice. Aortic arch lesion size in 20 week old mice was reduced by more than 50% compared to ovalbumin and PBS controls. Although the authors did not expect any undesirable immune problems using Treg specific for the whole HSP60 protein, in ublished at the same time compared oral and nasal tolerance induction to HSP65 using the same model and a high fat diet to induce atherosclerosis [114]. Tolerance induction again led to a reduction in lesion size, with nasal tolerance proving more successful. Mice showed a reduction in macrophage and T-cell infiltration and an increase in IL-10 production compared with control mice.

This provides an exciting opportunity for preventing or even treating atherosclerosis by vaccination as discussed below, and it will be interesting to see if specific

atheroprotective/atherogenic epitopes, which still have to be identified, will prove as efficient as the whole HSP60/65.

6 Role of the Innate Immune System

Macrophages enter the intima from the bloodstream as monocytes, with this migration representing an early step in atherogenesis, after T-cell migration [115, 116]. After differentiation into macrophages, these cells continue to play a crucial role in the development of an atherosclerotic lesion via several different mechanisms. They form foam cells (along with DC and SMC) by phagocytising modified (usually oxidised) LDL in a nonsaturatable manner by scavenger receptors [117–119]. This removes the cytotoxic oxLDL from the intima, but in doing so contributes strongly to the proinflammatory environment of the lesion by stimulating the expression of cytokines including IL-8 [120]. Macrophages are also a source of matrix metalloproteinases (MMP) which contribute to the vulnerable plaque formation by the degradation of stabilising extra-cellular matrix around the advanced plaque connective tissue cap [121]. Macrophages can also contribute to the oxidation of intimal LDL by the release of iron from phagocytosed erythrocytes [122]. On the other hand, phagocytosis of apoptotic cells, particularly prominent in advanced atherosclerotic plaques, by macrophages induces antiinflammatory factors such as IL-10 and TGF-β while repressing proinflammatory cytokines IL-1β, IL-8, IL-12 and TNF-α [123–126]. Impairment of the phagocytosis of apoptotic cells has been shown to lead to larger atherosclerotic lesions in mouse models, due to an increasingly proinflammatory environment [125, 127, 128]. However, it should not be overlooked that macrophages are also efficient antigen presenting cells, capable of activating $CD4^+$ T-cells via MHC Class II. As outlined in Section 5 above, this leads to T-cell proliferation and expression of a wide range of cytokines.

The complement system is a biological amplification system, comprising around 30 proteins interacting to form a cascade upon activation. They assist in the clearance and destruction of targets identified by antibody binding, via opsonisation or cytolysis of target cells, or by inducing the expression and release of cytokines and adhesion molecules. There are 3 pathways which lead to complement activation, the classical pathway, the lectin pathway, and the alternative pathway (recently reviewed in [129]). Activation of the classical pathway occurs when the inactive form of C1 binds to antigen bound antibodies or to CRP, and it's cleavage triggers the complement cascade. The lectin pathway avoids the need for C1 activation, with the next step in the cascade (activation of C4) occurring directly via mannan-binding lectin-associated serine proteases, which have indirectly bound to microorganism surface oligosaccharides via mannan-binding lectins. Alternative pathway activation can occur due to low levels of spontaneous C3 activation, and the subsequent binding and cleavage of Factor B. Both the classical and the alternative pathways have been shown to play a role in atherosclerosis, with markers and various activated complement components and regulatory proteins found in athero-

sclerotic lesions, while healthy arteries show no activation [130, 131]. Activation of complement components via necrotic cells and oxidised LDL has been shown in atherosclerotic lesions in vivo [132]. Due to the ability to clear apoptotic cells and immune complexes, the classical activation pathway has been found to protect against atherosclerosis, with humans deficient in this pathway being more prone to vascular disease and various other systemic autoimmune diseases such as systemic lupus erythematosus [133, 134].

Mast cells have been known to aggregate in atherosclerotic lesions for some time [135], although their exact role in atherosclerosis was not known. Suggestions had been made that mast cells lead to an increased vascular permeability and fibrosis, accelerating lesion development [19]. A recent study on LDL-receptor knockout mice crossbred with mice which were unable to develop mature mast cells has shown that mast cell derived IL-6 and IFN-γ promote atherogenesis [136]. These authors found that atherosclerotic lesion size in the mutant mice was greatly reduced compared to normal Ldlr-/- mice, and contained far fewer macrophages, CD4r T-cells, and apoptotic cells [136].

Toll-like receptors, members of the IL-1R superfamily, are critical to the innate immune response by acting as first line sensors of infection and inflammation. This occurs not by the detection of specific antigens, but by scanning patterns that are common across antigenic proteins from various species [137]. Activation of TLR-signalling leads to the expression of proinflammatory cytokines, various antimicrobial agents, and can induce the maturation of dendritic cells which may in turn initiate T-cell expansion and differentiation, linking the innate and adaptive immune responses. The pathogen associated molecular patterns (PAMPs) associated with the 11 human TLRs currently identified cover a wide range of bacterial and viral motifs. This includes lipoproteins and peptidoglycans (TLR2), viral double stranded RNA (TLR3), LPS and HSP60 (including human; TLR4), and bacterial CpG DNA (TLR 9). TLR are expressed on a wide variety of cells, including EC and various immune cells including dendritic and T-cells. Activation of TLR by ligand binding leads to a signalling pathway via the adaptor molecule MyD88, which requires further recruitment of IL-1 receptor associated kinases (IRAK) 1 and 4 along with tumour necrosis factor receptor associated factor 6. This pathway leads to the degradation of IKK and subsequent activation of NF-κB, and the transcription of a wide variety of genes, including proinflammatory cytokines and chemokines [138, 139]. TLR 3 and 4 are also capable of MyD88 independent signalling, activating interferon regulatory factor 3 and stimulating the expression of interferon and co-stimulatory molecules [140]. TLR2 activation leads to the expression of IL-8, IL-12, and IL-23, while TLR4 responses involve IL-10, IFN-β, and IL-12, underscoring the complexity of the innate immune response to various pathogenic determinants [141]. As the progression of atherosclerosis is an inflammatory/immune driven process, TLR provide a link between chronic infections and the inflammatory environment which promotes atherosclerosis. TLR expression has been shown in atherosclerotic plaques [142, 143], and TLR2 and 4 levels are found to be increased during lesion development in ApoE-/- mice [144]. Some studies have reported a reduction in the risk of atherosclerosis or coronary artery syndromes in humans with a Asp299Gly

TLR4 polymorphism [145, 146], however this effect was not reproducible in later studies with larger participant numbers [147–149]. Animal models with loss of function mutations of TLR4 or MyD88 showed significantly lower levels of atherosclerosis on an ApoE-/- background [150, 151], while TLR2 knockout mice showed only a slight reduction on a Ldlr-/- background [152]. Interestingly, treatment with the TLR2 agonist PAM3 on the same background resulted in a dramatic increase in lesion size, in a dose dependent manner [152]. Finally, since endogenous hHSP60 acts as a ligand for both TLR2 and 4 [153, 154], sHSP60 released from cells after stress and/or necrosis may activate TLR signalling, providing a connection to our HSP60 autoimmune hypothesis and alternative source of arterial inflammation.

7 Vaccination Against Atherosclerosis

As detailed in this review, the last 2 decades have experienced the collection of solid experimental and clinical data that speak for a primary role of immunological-inflammatory processes in the initial stages of artherogenesis as well as an important contribution to advanced lesions. Among the many candidate (auto) antigens HSP60 and oxLDL have received most of the attention. These two molecules therefore, are also prime candidates for the formulation of vaccines aimed at preventing or treating the disease via the induction of tolerance.

In earlier publications, oxLDL has been considered as a culprit autoantigen triggering an atherosclerosis-promoting immune response. As mentioned above, it later became clear that immunity against oxLDL is protective rather than atherogenic, most probably due to the elimination of this proatherogenic molecule from the circulation and tissue. Thus, immunisation of mice—both hypercholesterolemic wild-type and various types of knock-out models—leads to a significant reduction of atherosclerotic lesions [70, 155]. In this context it is of special interest that an IgM antibody produced by B1 cells against an oxidised phospholipid moiety shows an unexpected cross-reactivity with an epitope of pneumococcal polysaccharide. This observation will also open an additional avenue for protective intervention [156].

With regard to HSP60, the situation is more complicated due to the described extensive cross-reactivity between human and prokaryotic HSP60. Induction of oral or nasal tolerance against HSP60 protects mice from atherosclerosis induced by hypercholesterolemia and/or immunisation with HSP60 [113, 114]. The mechanisms underlying this successful tolerisation are not yet studied in detail but most probably involve the stimulation of Tregs. It is important to note that in humans tolerance against the whole HSP60 molecule would not be desirable because it may entail increased susceptibility to bacterial and parasitic infections. However, compared to the situation in rheumatoid arthritis where arthritogenic and arthritoprotective HSP60 epitopes have already been defined [157, 158], research is not yet as advanced in the field of atherosclerosis. Therefore, the endeavours of our and other groups are now focussing on the delineation of atherogenic and atheroprotective mammalian and mammalian-microbial cross reactive HSP60 T-cell epitopes

in addition to the already defined HSP60 B-cell epitopes [60, 61]. These epitopes will then be used for the development of a tolerogenic vaccine aimed at preventing and/or treating atherosclerosis, respectively.

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Immuno-Inflammatory Athero-Arteriosclerosis Induced by Elastin Peptides. Effect of Age

L. Robert and A. M. Robert

Contents

Abbreviations:

ECM	Extracellular matrix
SMC	Smooth muscle cells
ER	Elastin receptor
ROS	Reactive oxygen species
CFA	Complete Freund's adjuvant

Abstract: The emergence of cellular immunology in the second half of the 20th century triggered the interest of scientists and clinicians to explore the potential role of immune-mechanisms in degenerative chronic diseases, among others in athero- arteriosclerosis. These experiments were preceeded and encouraged by the important work of Klemperer, who coined the term collagenoses implying autoantibodies to collagen in such chronic diseases as disseminated lupus erythematosis and related chronic affections of connective tissues (Gardner 1965 for review). Several authors obtained reproducible vascular lesions similar to those observed in humans

L. Robert (✉) · A. M. Robert
Université Paris 5
Laboratoire de Recherche Ophtalmologique, Hotel Dieu Hospital
1 place du Parvis Notre Dame
75181 Paris cedex 04, France
Tel: 01 43 86 02 40
E-mail: lrobert5@wanadoo.fr.

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by immunizing rabbits with arterial wall homogenates. Using a fractional extraction procedure we could show that the major antigen responsible for this experimental immune-atherosclerosis was elastin, considered previously as nonantigenic. The more hydrosoluble macromolecular fractions of the vascular wall, although strongly antigenic, as judged from the production of precipitating antibodies, did not produce the same lesions with the same regularity and severity. Immunization of rabbits with highly purified elastin induced only a modest increase of circulating antibodies, but did produce arteriosclerotic plaques without any increase of dietary lipid administration. These results were completed and reinterpreted after the identification of the elastin-laminin receptor, activated by circulating elastin peptides by triggering a release of proteolytic enzymes and free radicals. The functional profile as well as the transmission pathway of this receptor, present on vascular cells and also on circulating white blood cells (WBC) was shown to change with age, loosing its physiologically relevant regulatory functions and preserving only its harmful effects. Circulating elastin peptides acting on the elastin receptor **(ER)** can induce vascular damage by upregulation of proteolytic (elastolytic) activity and reactive oxygen species (ROS) production. These reactions form a vicious circle with autoamplifying feedback mechanisms and age-dependent increase of the harmful effects on the vascular wall. A large number of human blood samples were tested for antielastin antibodies and also for elastin peptides. All blood samples contained both of these markers of the (auto)immune atherogenetic process involving the activation of the elastin-receptor, its uncoupling which results in the progressive increase of the degradative processes leading to the age-dependent amplification of the athero- arteriosclerosis. Elastin peptides were also shown to induce oxydation of LDL. This experimental model is an example of the delicate interplay of immune-triggered reactions with cell-signaling events during the development of athero- arteriosclerosis.

Keywords: Elastin • Aorta • Vascular wall • Antielastin antibodies • Elastases • Immune-atherosclerosis

1 Introduction

1.1 Historical Remarks

Although inflammation was recognized by Hippocrates and his school and defined by its cardinal symptoms (tumefaction, redness, heat, pain, and tissue dysfunction), its description in cellular terms had to await the birth of histochemistry. The invention of specific stains by Paul Ehrlich and others during the last decades of the 19th century, helped to designate the leucocytes as the essential cellular elements of the inflammatory process. With the development of clinical chemistry and biochemistry more and more molecular markers of the inflammatory process became available. Besides the increase of leucocytes in the blood and in tissues, the rate of red cell sedimentation, followed by that of the so-called acute phase glycoproteins

(haptoglobine, α_1 acid glycoprotein or orosomucoid) and of C-reactive protein were routinely determined and considered as the hallmarks of the inflammatory process. This same period was dominated by the birth and expansion of humoral immunity, dominated by the french and german schools (Pasteur, Roux, Behring and others). Refinement of methodology, with the routine practice of passive hemagglutination and the ELISA-methodology, an important progress could be achieved, enabling the detection and quantification of low concentrations of nonprecipitating antibodies, cytokines and other molecular players of the inflammatory process. Based also on refined methods of immunohistochemistry it became difficult to maintain the strict distinction between degenerative and inflammatory processes, examplified by the joint diseases. Arthrosis considered as a degenerative disease of articular tissue was more adequately designated osteoarthritis because of the inevitable development of the inflammatory process (Trentham 1984 for review). The possibility to create experimentally such articular pathology by immunizing with the major collagen component, collagen type II, of articular cartilage further blurred the frontiers between degenerative, inflammatory or (auto)immune mediated diseases. These conceptual and methodological advances prepared the way for the birth and expansion of cellular immunity, which dominated the field over the second half of the 20th century. This slowly emerging and finally dominating methodological and conceptual advances reached also cardiovascular pathology. During the last decades of the 20th century several teams entered the field of cardiovascular pathology, and introduced the above summarized methodology. These studies which will be described in this review revealed progressively the immuno-inflammatory nature of the athero-arteriosclerotic process also. Further progress came from the rapidly advancing field of cellular signaling. The study of receptors, agonists, antagonists, message transmission pathways considerably improved the understanding of the details of cellular-molecular immuno-inflammatory processes underlying such chronic, degenerative diseases as cardiovascular pathology. As however the lipid-based concepts continued to dominate the field of atherogenesis, the final junction between these different avenues of approach for the understanding of this family of diseases was difficult to achieve. Of great help for openminded scientists and physicians was the relatively rapid increase of the senior population over the last decades. Thanks to progress made in preventive and curative cardiovascular medicine, the fatal outcomes of cardiovascular pathologies were progressively postponed to later years of life, still remaining however the dominant cause of fatalities. This fact is certainly the motivation for experimental gerontologists to reassess the diverse contributing factors playing crucial roles in the development and progression of cardiovascular pathologies. Inflammation in particular was more recently recognized as an important factor in most age-related pathologies. A number of studies over the last decades clearly showed the importance of the inflammatory process as an omnipresent player in geriatric pathology. Cardiovascular pathology is no exception. Our laboratory actively participated in this progressive evolution of our concepts elaborated for the understanding of vascular diseases. We shall briefly review the successive stages of the above mentioned shifts of emphasis in the description and conceptualisation of vascular pathology, essentially its importance as an age related disease.

1.2 Arteriosclerosis and Atheromatosis

Progressive hardening, rigidification of the vascular wall, termed later arteriosclerosis, was recognized by early pathologists (Robert L. 1996, 1999a for review). These observations were based on autopsies and could not be easily generalized. The importance of nutritional factors in general and especially of cholesterol started to gain acceptance with the demonstration by Anitchkoff in the early decades of the 20th century of the induction of lipid infiltration and plaque formation in rabbits kept for several weeks on a cholesterol-enriched diet (Olsson 1987 for review). This model became the most widespread in laboratories of experimental medicine and formed the basis of the lipid-hypothesis of atherogenesis. The development of atherosclerotic plaques, as observed in human blood vessels at autopsy, could therefore be attributed to nutritional factors, cholesterol and saturated fat in particular. As however calcified plaques and lipid infiltration were regularly associated in human blood vessels, the term proposed by the german physician, Marchand: athero-arteriosclerosis was progressively adopted as more adequate to describe the human disease. The vast majority of scientists engaged in this field adopted the lipid hypothesis further comforted by the characterisation of lipoprotein-classes and during the second half of the 20th century by the description of the LDL-recognising receptor by Brown and Goldstein (Olsson 1987 for review). Some laboratories did however continue to explore avenues related to the immuno-inflammatory hypothesis.

2 Role of (Auto)Immune Factors

As mentioned in the introductory, historical section, immune-inflammatory factors were discovered as of crucial importance in some chronic diseases and especially in rhumatoid arthritis and osteoarthritis. Rapid progress in the field of extracellular matrix biology, in the characterisation of collagen(s), major components of connective tissues (more correctly of extracellular matrix, ECM) led to the identification of collagen type II as a major component of articular cartilage (Comper 1996 for review). Immunization with purified collagen type II was shown to induce osteoarthritic pathology. Autoantibodies to collagen type II were demonstrated in patient's sera (Trentham 1984 for review). Simultaneously several teams showed that immunization with arterial extracts could induce in rabbits athero-arteriosclerotic lesions.

2.1 Induction of Athero-Arteriosclerotic Lesions by Immunization with Arterial Homogenates

Apparently the first description of the production of athero-arteriosclerotic lesions in rabbits with homologous aorta-extracts was produced by a hungarian team (Szigeti I. et al. 1960, 1968). These results were obtained without excess

cholesterol administration. The severity of the lesions could be increased by prolonged protocols of immunization. The principally involved antigenic fraction was considered by these authors present in the saline-soluble fraction containing among other components a fraction with β-globulin mobility. Delayed-type tissue-allergic reactions could also be demonstrated. Similar lesions were created in rats by injection of rabbit sera immunized with aorta extracts. Total plasma lipids were shown to increase during immunization, similar to that found in cholesterol-fed animals (Szigeti I. et al. 1968 for review). Soon after these reports the french team of Scebat and Renais reported the production of immuno-atherogenic reactions in rabbits by immunization first with heterologous (rat) aorta extracts and later with homologous aorta extracts (Renais et al. 1968; Scebat et al. 1966, 1967;). In between White and Grollman (1964) produced periarteritis nodosa in rats also by immunization. The team of C.R.Minick at the New York Hospital (1966) also produced immuno-arteriosclerotic lesions by combining « allergic injury » and lipid-rich diet. – Altogether these experiments illustrated the possibility of an immune-mechanism underlying the atherogenetic process. As however all the above cited experiments were carried out with aorta-homogenates, the principal antigen(s) involved in this immune-atherogenic process remained to be determined. Some of the authors opted for the β-lipoproteins (LDL) of the blood-serum, present in the soluble aorta-extracts also as the principal atherogenic antigen.The hungarian team of S. Gero et al. (1959, 1960, 1967) induced lesions with anti-β-lipoprotein antibodies and proposed a variant of the immuno-atherogenic process based on these observations. The symposium organized by the French Atherosclerosis Society in 1964 in Bordeaux enabled the confrontation of these different views on the immune-factors involved in the atherogenic process. Our experiments, detailed in the next section, were also first presented at this meeting, proposing elastin as the main culprit as sensitising antigen and as the target of the immune-pathological process underlying athero-arteriosclerosis (Robert L. et al. 1967, 1968, 1970b, Robert A.M. et al. 1971).

2.2 Immune-Atherosclerosis Obtained with Purified Aorta-Extracts

The most important step to follow up on the above summarized results showing that active and passive immunization with crude aorta extracts could induce vascular lesions similar to human athero-arteriosclerosis was the reproduction of such experiments with purified aorta extracts. These experiments were performed in our laboratory and will be summarized in this section. Human and porcine aorta extracts were prepared, using lesion-free portions by a fractional extraction procedure used previously for other ECM-rich tissues as cornea and skin (Robert L.Parlebas, 1965). The soluble macromolecules were extracted with a 1 M Ca Cl_2 solution buffered to pH 8.0 with Tris/citrate (termed CTC-extract). The insoluble stroma remaining after several extractions in ice-cold buffer with the Ultra-turrax, was suspended in 2.7% TCA and heated to 90°C for 30 minutes to hydrolyze selectively insoluble collagen. The remaining stroma contains essentially the elastic fibers. Adhering microfibrillar

fraction (structural glycoproteins) were extracted by tourmixing the washed residue (0.9% Na Cl) in 8 M urea in presence of 0.1% mercaptoethanol. After centrifugation and washing in sterile 0.9% Na Cl the final residue analyzed as pure, insoluble elastin. Elastin was shown to be selectively hydrolyzed to large peptides when suspended in 1 M KOH in 80% (v/v) aqueous ethanol at 37°C for about 30 minutes (Robert L.Poullain, 1963). This large peptide solution (average MW about 70 kDa) was used for immunization. The other CTC, TCA and urea extracts were dialyzed and lyophylized. All these operations were carried out in sterile conditions, in the cold, on tissues delivered in dry ice. Rabbits (New Zealand-white or Fauve de Bourgogne), kept on rabbit-chow and fresh vegetables were immunized with 1–5 mg proteins in complete Freund's adjuvant, two injections weekly as described (Robert A.M. et al. 1971). After 4 weeks on this schedule the animals were left for 4 more weeks and received a final injection (i.v. or i.p.without Freund's adjuvant) with aluminium hydroxide as adjuvant. This was followed by testing animals for delayed hypersensitivity directly or using guinea pigs sensitized to the same antigens, as described (Robert A.M. et al. 1971, Jacob et al. 1984). Control animals received saline injections with or without Freund's complete adjuvant. Some rabbits received a cholesterol-enriched diet (1 g cholesterol in 7.5 ml peanut-oil homogenized with bran and barley). Titration of immune-sera was carried out with passive hemagglutination using glutaraldehyde-treated sheep erythrocytes. The antigens were fixed on treated erythrocytes using either a water-soluble carbodiimide or diazotated benzidine (Bing et al. 1967). The histological, histochemical and electronmicroscopic procedures were described (Robert A.M. et al. 1971). The CTC-extract contained a number of saline-soluble proteins and glycoproteins as shown by immune-electrophoresis and immune-diffusion according to Ouchterlony. The urea-extract contained several glycoproteins characterized by their size and glycan composition (Robert L. et al. 1967b, Robert A.M. et al. 1971). The κ-elastin solution had the typical amino-acid composition of purified elastin (Robert, Poullain, 1963). Purified elastin before and after urea extraction was also examined by electron microscopy (Robert B. et al. 1971) in order to demonstrate that urea-extraction largely eliminated the microfibrillar components. The amino-acid and glycan composition of the aorta fractions used as sensitizing antigens was described (Robert A.M. et al. 1971). As shown by immune-diffusion, antisera to the CTC-extract showed strong precipitation lines to several soluble macromolecules, not further characterized. They also showed faint precipitation lines to the urea- and κ-elastin fractions. Antisera to the urea-extract showed one strong and several faint precipitation-lines to the urea extract and one to three faint precipitation lines to κ-elastin. Anti-κ-elastin antisera also gave faint precipitation lines to the κ-elastin solution as well as to 2 to 3 faint lines to the urea extract. This precipitation lines, observed with antiCTC and anti-urea extract antibodies to the κ-elastin solution can be considered as a first indication of the presence of elastin peptides in the soluble fractions of human and porcine aortas. Up to this time elastin was considered as strictly insoluble. As shown by the passive hemagglutination tests, peak values of titrable antibodies were obtained at about 100 days after immunization, both to the urea extract and to κ-elastin. The titers fall than rapidely, suggesting an absorbtion of antibodies by the proper tissue-

antigens of the animals. Relatively high titers were obtained, about 10^{-5} for the urea extract and about $5x10^{-3}$ to κ-elastin (lowest hemagglutinating dilution of antisera). κ-elastin from human aorta gave higher titers than from porcine aorta. Cholesterol-fed animals showed low titers to all antigens tested (well below 10^{-2}). Delayed hypersensitivity reactions were regularly observed on the immunized rabbits as well as on guinea pigs sensitized to the same antigens. This can be taken as an indication that besides the humoral immune-reaction, immunizations with aorta-extracts did trigger also cellular immune-reactions. Total serum cholesterol did increase significantly 1-month after the onset of immunization (from 0,36±0,07 to 0,88±0,01 g/l in elastin immunized animals), and returned later to preimmunization values. β-lipoprotein (LDL) determined according to Burstein and Samaille (1959) did increase continuously with time in immunized as well as in control rabbit sera. This selective increase of circulating LDL might be attributed, besides immunization, to the diet and the aging of the animals. There were however significant differences between animals immunized with different aorta extracts. The strongest increase of LDL as compared to the starting levels (before immunization), were found in animals immunized with the urea-extracts (137–200% increase) followed by those immunized with elastin (without adjuvant!), 145% increase on the average. Immunization with elastin in complete adjuvant produced a less important increase (about 70%) compared to similar increase in nonimmunized control animals. The macroscopic and microscopic observations of the aorta of immunized rabbits revealed conspicuous modifications (Figs. 1–3). The most important modifications were observed on the aortas of animals immunized with pure elastin : lipidic-sclerous infiltrations covering most of the intimal surface, from the origin of aorta to the renal arteries, most obviously on the cross and around the ostia. Calcified plaques imitating egg-shell apparence, sometimes with aneurismal dilations were observed (Fig. 1). Migrating smooth muscle cells (SMC) were observed in the intima, accompanied by fragmentation of elastic lamellae (Figs. 2–4). Ultrastructural and histochemical studies confirmed the strong calcification of the fragmented elastic fibers. At some places necrotic modifications of the media were observed with SMC-s undergoing lysis. In all cases of immune-lesions a striking difference was observed with lesions produced by the high cholesterol diet. The cholesterol-induced lesions were similar to those described by a number of authors in other cholesterol-fed rabbits (Table 1). Differences were observed between the two strains of rabbits used. White rabbits were more resistent to immune-induced lesions than the fauve de Bourgogne strain as observed after several 1-month cycles of immunization followed by 1-month without. In our first experiments (Robert A.M. et al. 1971) about 40% of all the immunized animals presented the above summarized lesions with 25% presenting wide-spread macroscopic and microscopic lesions as those shown on Figs. 1–3. There were however important differences in the frequency and severity of lesions according to the antigen used (Table 1). Immunization with the soluble extract (CTC-extract) gave only infrequent lesions (1 out of 7 animals immunized) and only at the ultrastructural level. Immunization with elastin produced lesions both at the microscopic and macroscopic levels in more than 70% of the immunized animals. Immunization with the urea extract gave macroscopically detectable lesions, only in

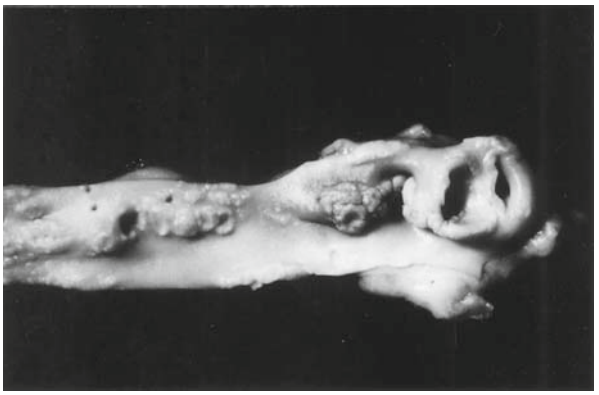

Fig. 1 Aorta of a rabbit immunized with κ-elastin from human aorta. Isolated or confluent fibrous calcified lesions covering most of the intimal surface. (From Robert AM. et al. 1971)

about 15% of animals, and microscopic lesions in about half of the immunized animals. These results suggested the presence of the most pathogenic antigen, supposed to be elastin, in all extracts, but in highly variable proportions: lowest concentrations in the CTC extract, somewhat higher concentration in the urea extract, and the highest in the purified elastin fraction. Further studies largely confirmed these observations (Jacob et al. 1984). The most severe microscopic and macroscopic lesions were seen in elastin immunized animals, followed by the urea extract. Such lesions included vacuolized, calcified endothelial cells (Fig. 2) above strongly dislocated, lyzed and calcified elastin lamellae. Table 1 shows a comparison of most frequent lesions seen in the aorta of animals immunized with the different aorta extracts. One of the crucial observations was the inverse relationship between the three aorta extracts used for immunization as far as the induction of precipitating antibodies and lesion-induction is concerned. The soluble (CTC) extract induced high titers of precipitating antibodies and only infrequent and relatively mild lesions. Purified elastin, wether used in its insoluble, fibrous form or as soluble, κ-elastin, did induce regularly the most severe lesions, but gave only low titer hemagglutinating or precipitating antibodies, the urea extract occupying an intermediary position. The low titer of circulating antibodies could best be explained by their adsorption on elastin fibers and was confirmed by the fixation of immunofluorescent anti-rabbit-IgG antibodies

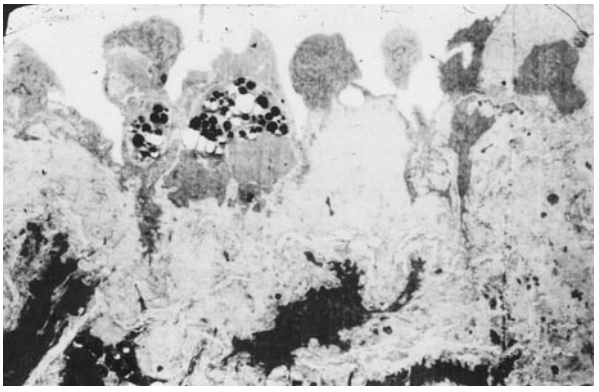

Fig. 2 Intimal portion of rabbit aorta immunized to urea extract of porcine aorta. Electron microscopy, 3,200x. Notice the granular infiltrate of strongly modified SMC-s, fragmented, lyzed and calcified elastic lamellae and vacuolized and calcified endothelial cells. (From Robert AM. et al. 1971)

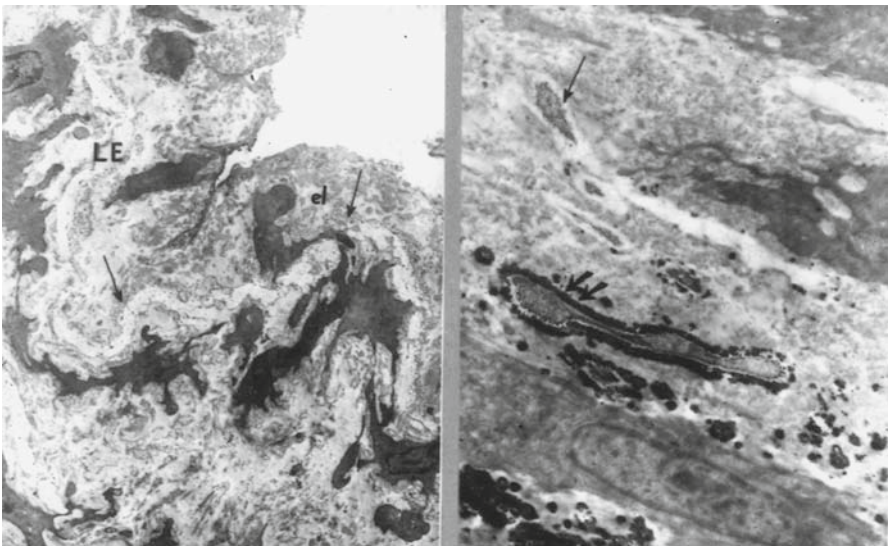

Fig. 3 Modified, fragmented, lyzed and calcified elastic lamellae (LE) in the thoracic aorta of rabbits immunized with human κ-elastin. Fibrillar deposits (arrows) and strong perielastic calcification (double arrow; Electron microscopy by Grosgogeat Y. in collaboration with Robert A.M. et al. 1971)

on elastic fibers (Jacob et al. 1984; Robert L. et al. 1967, 1968, 1970b). The predominant localization of the lesions at the level of elastic fibers was certainly the most conspicuous observation: fragmentation and calcification. Degenerescent modifications of endothelial cells and SMC-s was also a constant observation. Extra-

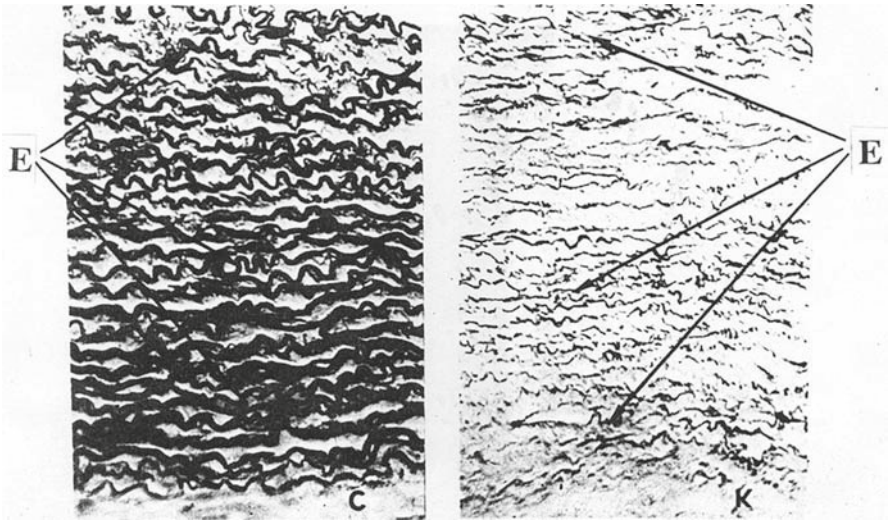

Fig. 4 Fragmentation of elastic lamellae in aortas of rabbits immunized with κ-elastin (*right*, K) as compared to a control aorta (*left*, C). E = elastic fibers. (From Jacob et al. 1984)

Table 1 Macroscopic, microscopic and ultrastructural lesions of the aortas of rabbits immunised with aorta extracts

Antigen injected	n animals with visible lesions	Nature and intensity of lesions		
		Macroscopic	Microscopic	Ultrastructural
Controls NaCl 0,9% + adjuvant	0 out of 5	No lesions	No fibrosis Normal elastic lamellae	No lesions
CTC extract	1 out of 7	No Calcified lesions Nor plaques	No Fibrosis of intima or media Elastic lamellae intact	No necrotic SMC-s No intimal hyperplasia
Urea extract	8 out of 12	Diffuse lesions covering Part of the intimal surface	Necrotic plaques in media Fibrosis Calcified elastic lamellae	Fibrotic infiltration of the intima Elastic lamellae fragmented, fibrillar, calcified Degenerescence of cells in media
κ—elastin	5 out of 7	Heavy lesions covering the whole intimal surface Strong calcification	Fibrosis of intima; elastic lamellae lysed, fibrillated, calcified; medial necrosis; SMC-s disoriented	Medial fibrosis Calcified deposits in elastic lamellae and collagen fibers
Cholesterol feeding	2 out of 4	Soft lipidic lesions at the cross No calcification	Lipidic infiltrate of intima Foam cells, fibrosis	No calcification Endothelial cells with lipid infiltration; intercellular fibrosis Rupture of elastic lamellae

(modified from Robert A.M. et al. 1971).

cellular calcium-containing crystals could also be observed occasionnally. The macroscopic and microscopic-ultrastructural lesions were quite similar to those observed in human athero-arteriosclerosis, but quite distinct from those produced by the cholesterol-rich diet. Although a more or less important and transitory increase in β-lipoproteins (LDL) was observed in immunized animals, we also did observe unexpectedly an increase of antielastin antibody titer in the sera of cholesterol-fed animals, although the titers remained relatively low ($\leq 10^{-2}$). Further experiments on the elastin receptor (ER) of vascular cells confirmed the role of elastin peptides in the lipid-induced lesions also (*See* later). Some years later these experiments were repeated in our laboratory, using only κ-elastin as the sensitising antigen (Jacob et al. 1984). These experiments will now be described.

2.3 Extension of Elastin-Immune-Pathology to Pulmonary Vessels, Metabolic Effects

The above described experiments were repeated using this time only κ-elastin of high molecular weight purified by gel filtration (≥ 70 kDa, designated κ_1—elastin) from bovine ligamentum nuchae-elastin as the immunizing antigen with New Zealand white rabbits, carried out essentially as in the first experiments (for methodological details *See* the original publication, Jacob et al. 1984). This time however besides the aorta, detailed investigations were carried out on the small arteries in the lung parenchyma also. The macroscopic, microscopic and ultrastructural investigations were completed by experiments on the biosynthetic capacity of aorta-wall explant cultures using ^{14}C-lysine and ^{14}C-glucosamine incorporation. Another innovation as described in more detail in the next section was the determination of elastase-type endopeptidase activity in aorta extracts from control and immunized animals. The presence of such activity in human aorta extracts was previously described in our laboratory (Hornebeck et al. 1975b; B. Robert et al. 1974b;) as will be detailed below. This activity could explain the presence of soluble elastin-peptides in the buffer-soluble aorta extracts, suspected in our first experiments. The biological properties of elastin peptides started to be studied in several laboratories and will

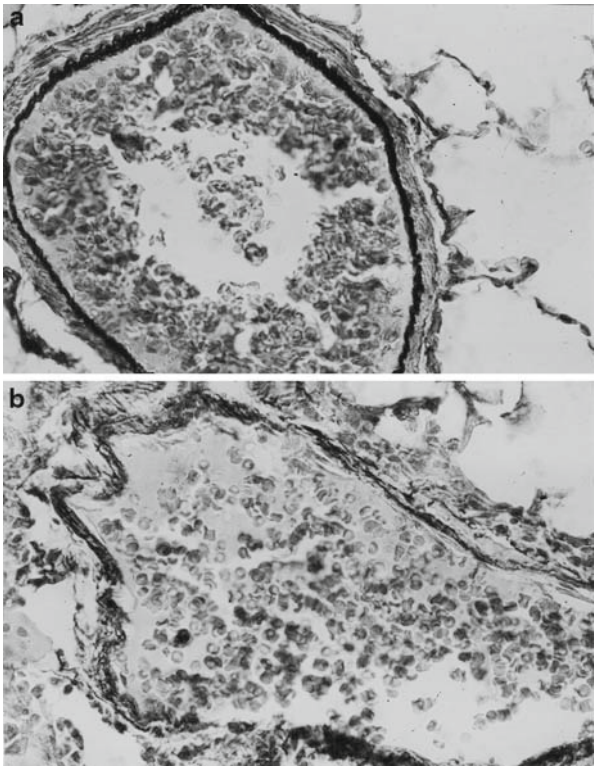

Fig. 5 Histological appearance of rabbit lung arterioles in control (**a**) and in κ-elastin immunized (**b**) rabbits. (Modified from Jacob et al. 1984)

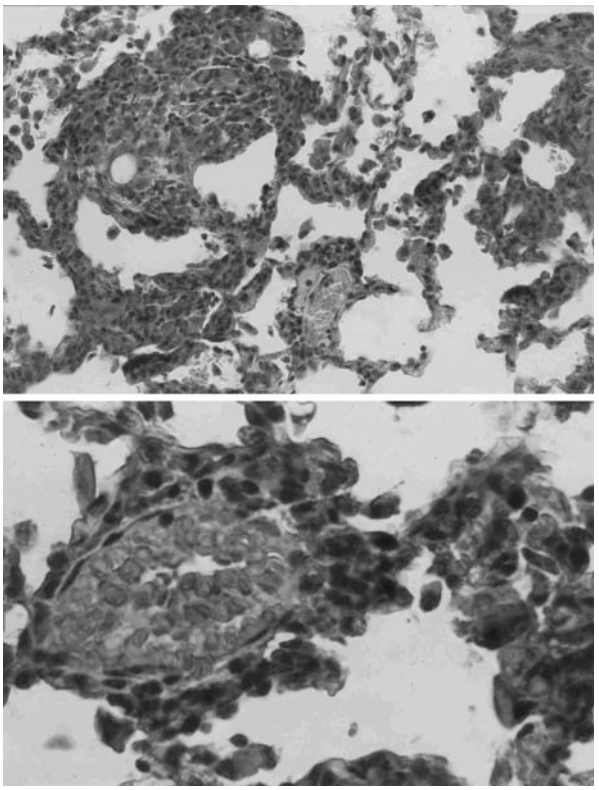

Fig. 6 Granulomatous lesion with vasculitis in the lungs of κ-elastin immunized rabbit. (Unpublished results from Chantal Lafuma in the author's laboratory)

be described in the following sections. These relatively recent results extended the horizon of our investigations and triggered a more complete search for pathological effects produced by elastin immunization as well as by elastin peptides. This time all rabbits immunized to κ-elastin developed typical vascular lesions at the macroscopic and microscopic level similar to those seen in our first experiments described in the previous seection. The histological study of the lungs of the immunized rabbits revealed the presence of granulomatous lesions, absent in the control animals (Figs. 5, 6). The most conspicuous modifications were however the lysis of elastic laminae in the large vessels as well as in the walls of small lung-vessels (Figs. 4, 5). This time the loss of continuity of elastic fibers was quantitatively assessed by computerized image-analysis. A pronounced fragmentation of elastin fibers could be demonstrated as shown on Figure 4. No such fragmenhtation was seen in vessels of control animals injected only with complete Freund's adjuvant or with BSA in complete Freund's adjuvant. A curious observation was the more pronounced fragmentation of the elastic lamellae in the outer segment of the media than in its inner segment, near the intima. The SMC-s of elastin-immunized animals showed striking morphological changes and random orientation, comparable to those seen in our first experiment (Figs. 2, 3). Their number was also decreased. The average length of elastic lamellae were about 13-times shorter in elastin-immunized aortas as compared to controls receiving adjuvant injections with or without BSA.

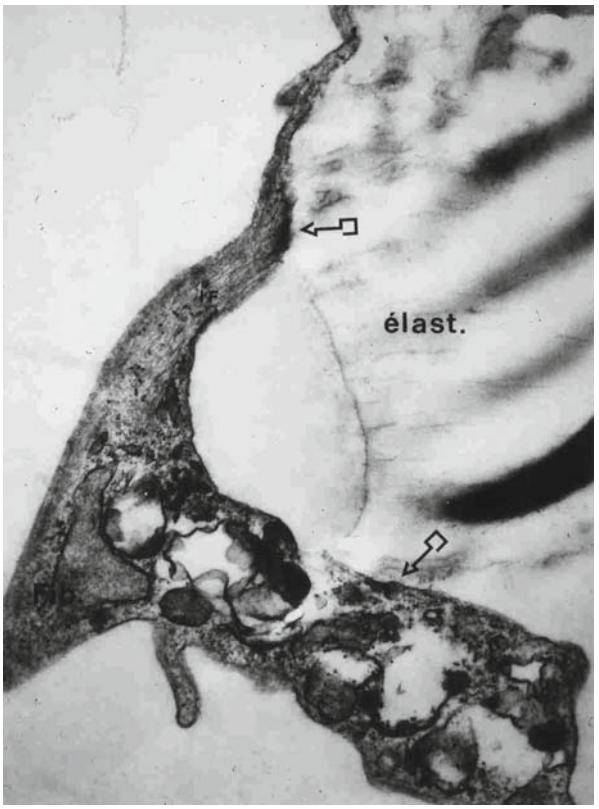

Fig. 7 Strong adhesion of a fibroblast to a purified and micronized elastic fibril under the electron microscope. No such adhesion with SMC-s from elastin-immunized animals was observed (modified from Perdomo et al. 1994)

This proportion when calculated separately for the internal part of the media gave a ratio of only four times shorter elastin segments for the inner part of the media and a much more pronounced fragmentation, 40-times as compared to controls in the outer half of the media. In the lungs of the elastin-immunized animals intense granulomatous lesions were seen as well as a strong elastolysis in the walls of small lung-vessels (Figs. 5, 6.). The granulomatous lesions contained giant cells and eosinophil leucocytes. On the ultrastructural level the findings were similar to those described in our first experiments (Robert A.M. et al. 1971). Electron microscopy confirmed the alterations of SMC-s and elastic lamellae which were fragmented, disorganized with an increased density of the microfibrillar components (Fig. 3). Endothelial cells were enlarged, vacuolated and calcifyed as in the first experiments (Fig. 2). One of the striking observations was the loss of continuity of elastic fibers or close contact between SMC-s and elastic lamellae, (Fig. 7) clearly seen in control aortas. The increased titer of antielastin antibodies in the κ-elastin immunized rabbits could be confirmed by passive hemagglutination, the titers were however lower than in the first experiments (10^{-2}–10^{-3}). The adsorption of these antielastin antibodies to purifyed, micronized elastin fibers could be confirmed by immuno-peroxidase staining using sheep anti-rabbit Fb antibodies. No such reaction was seen with sera of animals immunized to BSA in Freund's complete adjuvant. The same reaction,

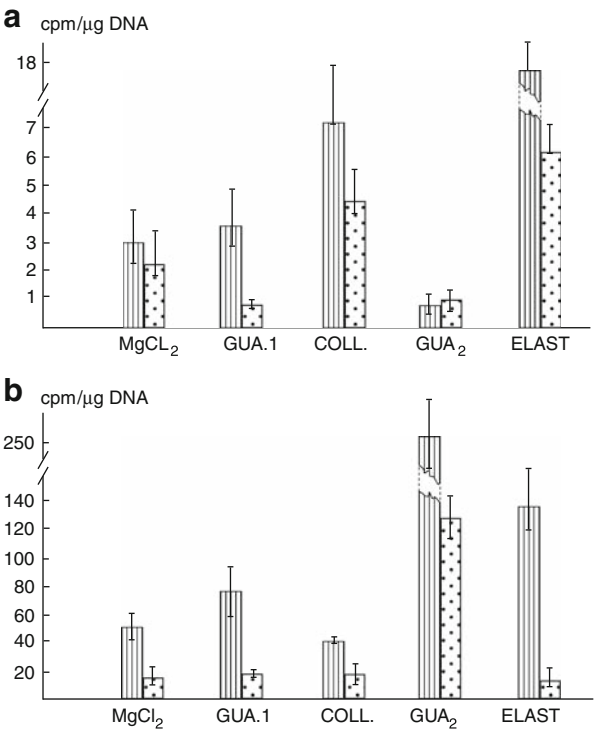

Fig. 8 Incorporation of ^{14}C-lysine (**a**) and ^{14}C-glucosamine (**b**) in proteins of arterial wall extracts. Bars with lines: control group (CFA); bars with points: κ_1-elastin immunized rabbit group (κ_1-CFA). Ordinates: radioactivity incorporated in each extract, expressed as cpm/µg DNA. Abscissa: $MgCl_2$: 2M $MgCl_2$ extract; GUA_1, first guanidinium chloride extract ; COLL , collagenase extract; GUA_2, second guanidinium chloride extract; ELAST, elastase extract. The bars represent the SEM for the average value of each extract (For details *See* Jacob et al. 1984)

adsorbed autologous IgG on aorta elastic fibers could be demonstrated in the κ-elastin immunized rabbits also.

Figure 8 shows the results of radioactive lysine and glucosamine incorporation by aorta explant cultures, results beeing expressed as cpm per mg DNA. Incorporation was strongly decreased in the aorta explants from the κ-elastin immunized animals as compared to control animals injected with the complete adjuvant alone. Another important observation was the increase of elastase-type endopeptidase activity of the aorta extracts of κ-elastin immunized animals (Table 2). N-succinyl-ala_3-PNA was used as the chromogenic substrate, relatively specific for elastase-type endopeptidases (Bieth, 1978). These experiments largely confirmed and extended our first observations (Robert A.M. et al. 1971) on the pathological modifications of large and small elastic vessels in animals immunized to human, porcine

Table 2 Elastase-type endopeptidase activity of soluble extracts of aortas from rabbits immunized or not with κ-elastin

Nature of sera	Activity	Significance
Control	130±23.4	
Immunized	434±111.2	$p<0.01$

The endopeptidase activity of sera was determined with N-suc-ala_3-PNa and expressed as nanograms of porcine pancreatic elastase equivalents per mg of DNA. The figures of the table are averages of 5–7 animals. The statistical significance of the results was determined according to the Mann and Whitney distribution free test (Modified from Jacob et al. 1984).

or bovine elastin-peptides. All treated animals showed characteristic macroscopic, microscopic and ultrastructural modifications with conspicuous fragmentation of elastic lamellae, modifications of endothelial cells and SMC-s and also strong calcification. Further experiments were carried out in order to specify the individual aspects of these moditications at the cellular and molecular levels.

3 Cellular-Molecular Mechanisms Involved in the Immune-Inflammatory Vascular Pathology

The above described experiments confirmed the possibility of the induction in rabbits of a macro and microvascular pathology reminiscent of arterio-atherosclerosis by immunization with elastin. Peptides derived from highly purifIed fibrous elastin, κ-elastin was shown to be the most efficient (if not the only) inducing antigen. The humoral immune reaction could be confirmed by the passive hemagglutination test and by immunodiffusion. There was however an inverse relationship between antibody titers and efficiency of lesion-induction when consecutive aorta extracts were used.The ultrastructural studies revealed severe modifications of cells and ECM-components, mainly of elastin. Among the most conspicuous modifications was intra- and extracellular calcification, obvious even at macroscopic examination of longitudinally opened aortas of elastin-immunized rabbits. It remained to elucidate the underlying cellular and molecular mechanisms. Some of these experiments will now be described.

3.1 Degradation of Elastic Fibers

One of the most conspicuous modifications produced by immunization with elastin was the pronounced degradation of elastic fibers of the large and small blood vessels. It could be shown that this is at least partially the result of an autoamplifying vicious circle mediated by the action of elastin peptides on the ER. As shown on Fig. 9 the addition of elastin peptides at concentrations shown to be present in the blood serum (*See* later) produced a pronounced upregulation of the production of elastase-type

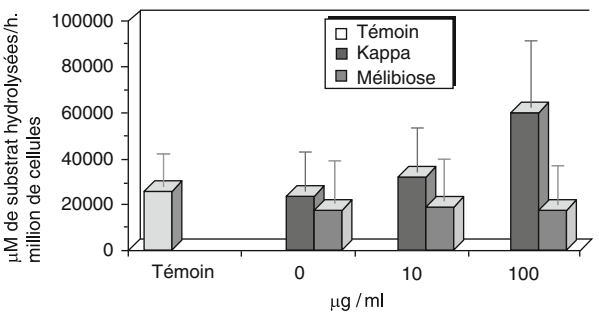

Fig. 9 Increase of elastase-type endopeptidase activity in fibroblast cultures in presence of increasing concentrations of κ-elastin (0,10 ad 100 µg/ml). Addition of melibiose, an ER antagonist inhibits this upregulation of elastase activity. (Modified from Archilla Marcos, Robert L. 1993)

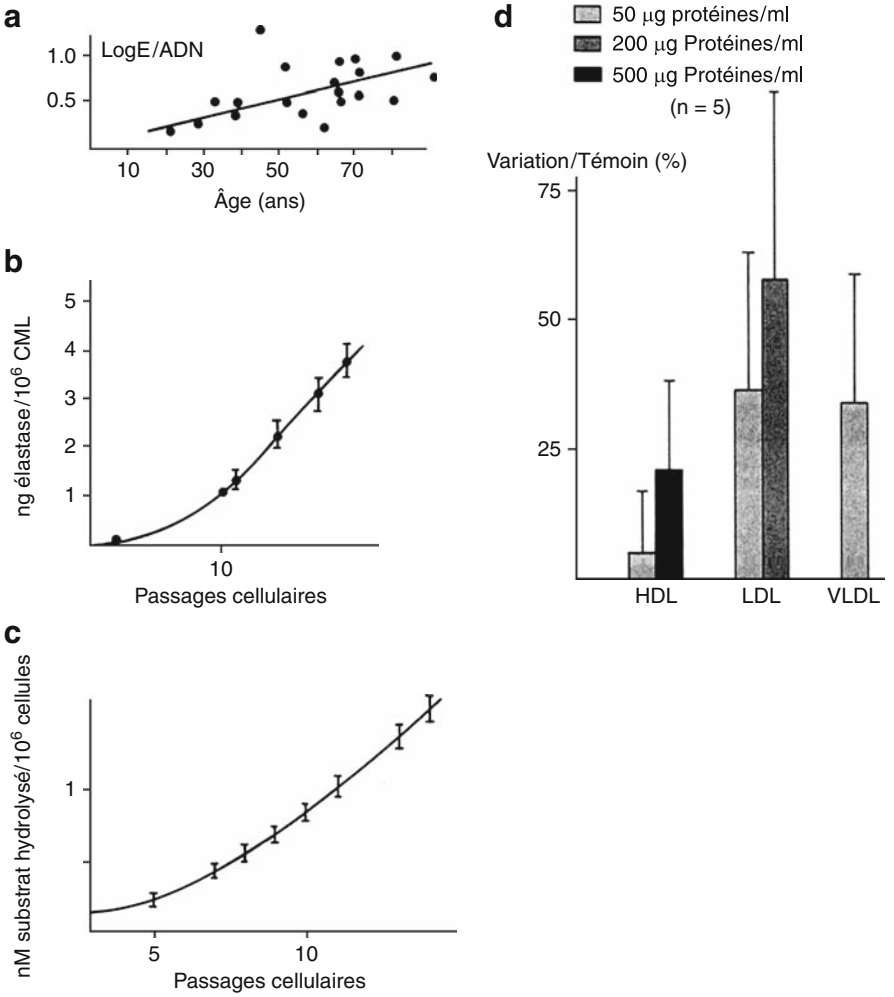

Fig. 10 Elastase type endopeptidase activity determined with N-suc-ala$_3$-Pna as substrate. **a**: in human aorta extracts. Abscissa: age in years, ordinates: log elastase activity per cell (DNA); **b**: in successive passages of aorta smooth muscle cells. Abscissa: passage number, ordinates: elastase activity given as ng equivalent of pancreatic elastase; **c**: in successive passages of human skin fibroblast cultures. Abscissa: passage numbers, ordinates: nM substrate hydrolyzed / 10^6 cells; **d**: effect of human lipoproteins added to SMC-cultures. For other details *See* text and Robert L. et al. 1986

endopeptidases (Robert L. et al. 1986) essentially of MMP-2 and MMP-9 (Archilla-Marcos, Robert L.1993). This increase of elastase production could be inhibited by lactose and melibiose, antagonists of the ER. It could also be shown during these studies that aging, both chronological and in vitro (increasing passage number) produced an increased expression of elastase-type endopeptidases (Fig. 10). These experiments, repeated over the years, suggested a mechanism for the progressive degradation of vascular and pulmonary elastic fibers. Using an ELISA-procedure,

the concentration of circulating elastin peptides could be determined in a large number of normal and pathological human sera (Bizbiz et al. 1997; Fülöp et al. 1989a). Their level followed a Gaussian curve with an average concentration in the μM range, largely exceeding the K_D value of the ER, shown to be in the nanomolar range (Fülöp et al. 1989b; Robert L. et al. 1989). In elastin-immunized animals these processes appear to be exagerated by the action of soluble immune complexes on monocytes and lymphocytes able to trigger an increased release of elastin degrading endopeptidases. The presence of antielastin antibodies in all human sera tested suggests a similar mechanism in human also (Fülöp et al. 1989a, b). The selective and irreversible adsorption of elastases on the surface of elastic fibers largely limits the possibility to counteract this process by elastase inhibitors (Robert B. et al. 1974a).

3.2 Calcification of Elastin

The intense calcification of elastin fibers in the immunized animals, seen macroscopically and on the electron-microscopic preparations, could be confirmed by specific histochemical methods (Van Kossa staining) and direct chemical determinations. A partial explanation of this strong affinity of elastin for calcium came from physicochemical studies of Dan Urry (1980) showing that the β-turns of elastin represent high affinity fixation sites for calcium. Earlier studies by Max Burger (1947) and Lansing (1959) revealed the progressive Ca-fixation in elastic blood vessels in a diffuse fashion, and still present in elastin purified by heating to 100°C in 0,1 N NaOH, the standard procedure for purifying elastin (Robert L. et al. 1985 for review). Moreover it was shown that calcium fixation on elastin strongly potentiates its affinity for lipids (determined by using ^{14}C-cholesterol (Jacob et al. 1983; Hornebeck, Partridge, 1975a) and vice versa, lipid fixation potentiates Ca-fixation. As β-lipoproteins (LDL) increased during the first phase of elastin-immunization, this also could contribute to Ca-retention in elastin. We have to mention here the demonstration by the team of J.L.Beaumont the role of anti-LDL antibodies in the development of atherosclerosis (Beaumont 1965, 1969, 1970, Beaumont et al. 1965, Beaumont, Beaumont, 1968). This team demonstrated also the importance of anti-heparin antibodies in atherogenesis (Beaumont, Lemort 1974, Buxtorf et al. 1981, Lorenzelli-Edouard et al. 1980). The work of Bihari-Varga and Gero pointed also on the potential role of LDL and anti-LDL antibodies on the atherogenic process (1966, 1984, 1986). This team pionieered also the recognition of the acid polysaccharides (glycosaminoglycans, proteoglycans) of the vascular wall in the retention of lipoproteins (Bihari-Varga, Gero, 1966). These two, essentially postsynthetic processes, calcification, lipid fixation confirmed by direct analysis of lipid classes in purifyed human aorta elastin by Claire et al. (1976) largely explained the progressive loss of elasticity and increased susceptibility to degradation of vascular elastic fibers.

3.3 Role of the Elastin Receptor

The demonstration of an elastin-recognising cell-membrane receptor started in our laboratory with the observations on the capacity of cells to strongly adhere to micronized elastin fibrils, as shown by time lapse video-microscopy (Hornebeck et al. 1986). Addition of elastin peptides to cells (fibroblasts and vascular SMC-s) was supposed to compete with fibrous elastin and inhibit elastin fiber fixation on cells. The opposite effect was observed, low concentrations of κ-elastin strongly increased the speed of adherence of cells to elastic fibers (Groult et al. 1998). This effect could be attributed to the induced synthesis of a membrane glycoprotein of 120 kDa termed elastonectin (Hornebeck et al. 1986). These initial experiments were followed by a series of other experiments aimed to the exploration of the physio-pathological roles of the elastin-receptor. Using immune-histochemical procedures, the presence of the elastin-receptor could be demonstrated on vascular cells, endothelial cells, SMC-s, fibroblasts as well as on WBC-s, monocytes, PMN-s and lymphocytes (Faury et al. 1995, 1998a; Jacob et al. 1987a, Perdomo et al. 1994, Péterszegi et al. 1997c). Most importantly, the elastin-receptor could be demonstrated on monocytes and lymphocytes inside the human atherosclerotic plaques obtained by endarterectomy (Péterszegi et al. 1997a). A variety of tumor cells did also exhibit the ER (Timar et al. 1995). All these cells, mobile and sessile, found in the vascular wall might therefore contribute to the ER-mediated upregulation of elastolytic protease production. It could be shown also during a long collaboration with T.Fülöp and his team, that the activation of the ER contributes by still other mechanisms to the progression of vascular lesions. One of these mechanisms is the elastin-peptide triggered release of superoxide (Fülöp et al. 1989b) suggesting a free radical (ROS-mediated) contribution to the vascular-cellular lesions. Another significant observation was the demonstration of accelerated oxidation of LDL by activation of the ER (Fülöp et al. 2005). It also could be shown that aging accelerated these harmful effects mediated by the ER. In cells (monocytes, PMN-s) obtained from old individuals, the normal transmission pathway of ER, as shown to function on young cells (Fülöp et al. 1990a; Varga et al. 1988, 1989) did no more function as shown by the inefficiency of pertussis toxin to block the transmission pathway mediated by a Gi protein in young cells. Free radical release was however maintained and even amplified. These observations suggested an uncoupling of ER in old cells from its normal transmission pathway. This uncoupling results in the exacerbation of the harmful effects mediated by the receptor and loss of its physiologically useful functions, as NO-mediated vasodilation (Faury et al. 1997, Fülöp et al. 1992) and inhibition of cholesterol synthesys by monocytes (Varga et al. 1997). Most of these experiments as well as others pertaining to the role of ER in the malignant process were reviewed recently (Labat-Robert J., Robert L. 2007). ER-mediated mechanisms play an important role in the inflammatory process also, which accompanies the immune-induced pathologies. Elastin peptides were shown to be chemotactic to WBC-s, monocytes especially (Antonicelli et al. 2007 for review). Activation of the ER on PMN-s and monocytes triggers the release of proinflammatory enzymes

and cytokines (Antonicelli et al. 2007 for a review). ER-triggered reactions include endothelial iNOS upregulation and NO-dependent vasodilation (Faury et al. 1998a, b). As mentioned above, ER-NOS coupling decreases with age with a steady age-dependent loss of vasodilation by elastin peptides (Faury et al. 1997). Besides the ER-triggered proinflammatory reactions, soluble immune-complexes formed by the reaction of antielastin antibodies and circulating elastin peptides might well contribute also to the harmful effects produced by immunization with elastin. These rapidly summarized experiments substantiated the claim for an important pathogenetic role of the elastin–antielastin system in the genesis of athero-arteriosclerosis (Fülöp et al. 2001; Robert L. 1999a, b).

4 Extension to Human Pathology

The first experiments on immune-atherosclerosis were performed on human sera with the demonstration of antielastin antibodies present in all sera tested, using the passive hemagglutination method (Stein et al. 1965). Although the titers were relatively low (from 1/2 dilution to 1/512 in these first experiments) it could be shown that sera from 68 atherosclerotic persons were in the lower range (1/2–1/32 dilution still giving agglutination) attributed to the enhanced adsorption of antielastin antibodies on degrading elastin fibers offering a larger surface for antigen–antibody complex formation. Such complexes were demonstrated on fibrous elastin (Jacob et al. 1984, 1987b). The anti-elastin antibodies appeared at about 20 years and disappeared after 80 years (Stein et al. 1965). Surface area measurements on purified elastin using radioactive Krypton (Robert L. et al. 1970a, 1971) revealed a relatively large specific surface of elastin fibers, favoring interaction (adsorption) with soluble molecules. These results were further confirmed by microcalorimetric adsorption studies (Robert L. et al. 1971). Later experiments (Fülöp et al. 1989a) confirmed the presence of antielastin antibodies, by direct titration of IgG and IgM type antibodies, in a larger number (265) of human sera, normal and pathological, mainly athero-arteriosclerosis of the legs, ischemic heart disease, stroke, diabetes, hyperlipidemia type II/b and IV and hypertension. No obvious correlation was found with age. IgG-type antielastin antibodies were elevated in obliterative arteriosclerosis of the legs and ischemic heart disease. No such modifications were seen in the IgM-type antibodies. Both types of antibodies were however decreased in type IV hyperlipidemia. Figure 11 shows the age-dependent evolution of the above summarized determinations of antielastin antibodies in human sera. More details on the specificity and sensitivity of the ELISA method used can be found in the original publication (Fülöp et al. 1989a). Circulating elastin peptides were also determined by an ELISA-procedure on about 1,500 human sera (Bizbiz et al. 1997; Fülöp et al. 1990b). These last experiments performed in collaboration with epidemiologists on a study on the role of vascular aging on brain aging (the EVA-study, Bizbiz et al. 1996) confirmed also the correlation between carotid artery wall thickness and plaques with the elastin-elastase parameters. Elastase-type endopeptidases were also demonstrated in a large number

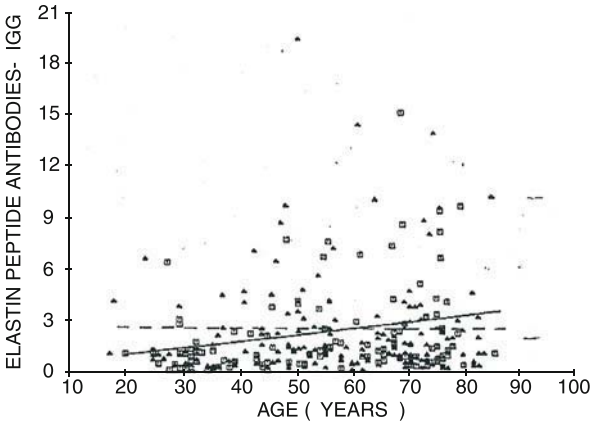

Fig. 11 Correlation between anti-elastin peptide antibody concentration (μg/ml) of IgG type and the age of subjects. males (r = 0.19, n = 117)(—), females (r= 0.01, n = 166) (— —)

of human sera (Table 3, Hornebeck et al. 1983, Bihari-Varga et al. 1984, Bizbiz et al. 1996). Monocyte-macrophage elastase-type activity was shown to be upregulated by cholesterol and by proinflammatory cytokines (Rouis et al. 1990). It should be mentioned however that after screening a large number of synthetic elastin peptides by monoclonal and polyclonal antibodies, it could be shown that according to their structure they presented a variable reactivity to antibodies used for their detection and quantification (Wei et al. 1993). Further confirmation of the role of the elastin-immune system and of the cenral importance of ER-mediated processes came from the above mentioned demonstration of ER-exhibiting mononuclear cells in freshly excised human endarterectomy samples (Péterszegi et al. 1997a). It also could be shown that human helper (CD4) and memory (CD45R+) lymphocytes when cultured in presence of elastin peptides exhibit inducibly the ER as shown by flow-immunocytofluorimetry (Péterszegi et al. 1997c). Addition of increasing concentrations of elastin peptides upregulated in a dose-dependent fashion the expression of a serine-elastase identical to PMN-elastase (shown by inhibition with monoclonal anti-PMN-elastase antibody) followed by cell death (Péterszegi et al. 1999; Fig. 12). The crucial role of the ER could be demonstrated in these experiments also by the inhibition of cell death in presence of the antagonists of the ER, lactose and melibiose (Robert L. 1999a, b). It. also could be shown that circulating lymphocytes isolated from the blood of elderly patients suffering from denutrition and dementia exhibited an increased elastase and cathepsin G activation mediated by the ER (Péterszegi

Table 3 Elastase-type endopeptidase activity of normal and pathological human sera

Nature of sera	Activity	Significance
Control	78.1±41.9	
Atheroma	91.1±63.8	N.S.
Emphysema	237,2± 133.8	$p<1\times10^{-8}$

The endopeptidase activity of sera was determined with N-suc-ala_3-PNa and expressed as μg/ml pancreatic elastase ±SD. The statistical significance of the results was determined according to the Mann and Whitney distribution free test (Modified from Hornebeck et al. 1983).

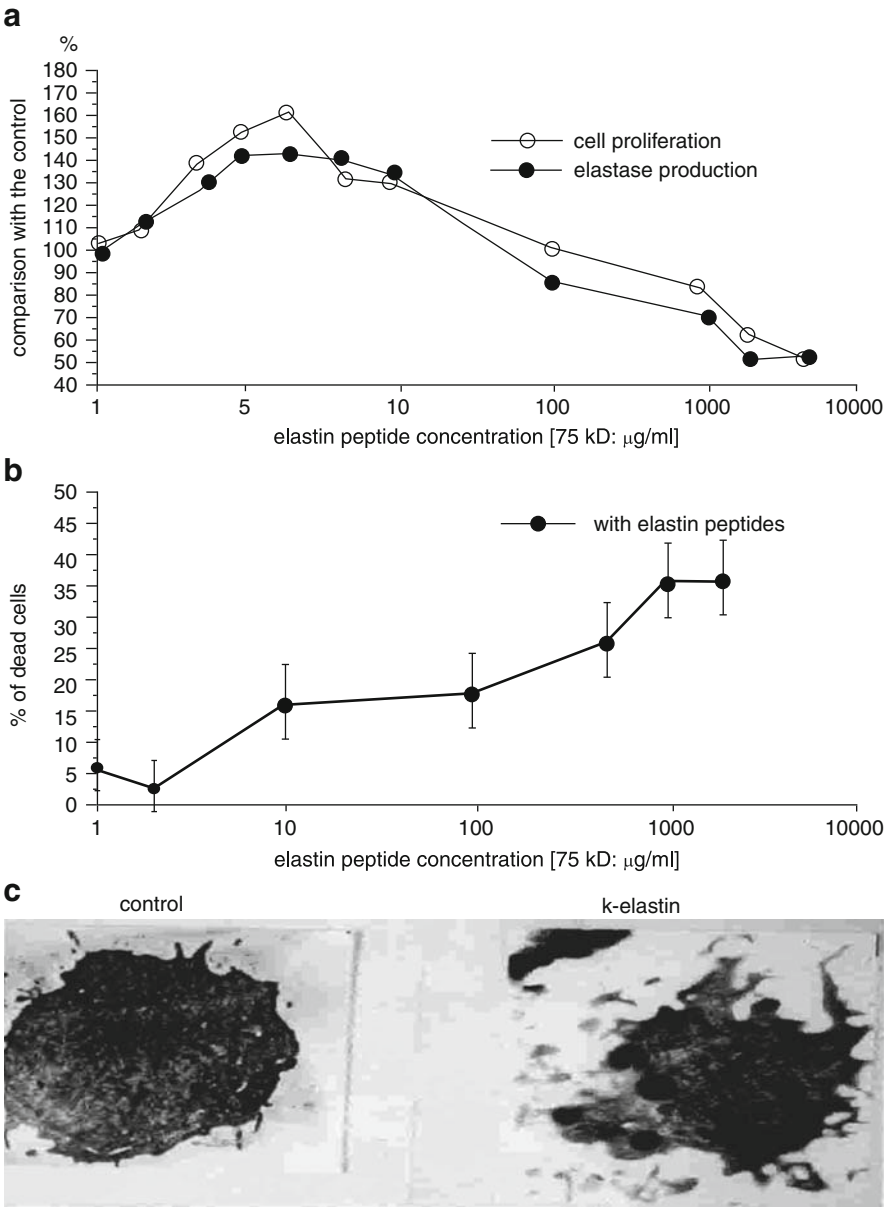

Fig. 12 Upper Fig. **a**: Human lymphocytes cultured in presence of increasing concentrations of κ-elastin (in log conc.μg/ml on the abscissa). -o- : modification of cell proliferation and of elastase production -●- as a function of κ-elastin concentration. **b**: increase of cell death with increasing κ-elastin concentration, as % of total cells. **c**: Electron microscopy of a normal lymphocyte from the above cultures (control) and a lymphocyte with apoptotic bodies (κ-elastin) (modified from Péterszegi et al. 1999)

et al. 1997b). Another finding in favor of the above hypothesis, connecting the immun-hypothesis to the lipid-theory of atherogenesis came from the demonstration of an increased concentration of elastase-type endopeptidases in aorta-extracts from cholesterol- fed animals (Jacob et al. 1982) confirmed by in vitro experiments showing that addition of human LDL and VLDL (but not HDL) to vascular SMC-cultures strongly increased the production of elastase-type endopeptidases (Fig. 10; Bourdillon et al. 1984).

The pharmacological consequences of the above described immuno-atherosclerotic process were also investigated. It could be shown that immunization of rabbits with elastin strongly increased SMC-membrane permeability, as shown by the increase of ouabaine-insensitive $^{22}Na^{+}$ efflux, the ^{86}Rb efflux (indicator of K^{+} efflux) and the $^{45}Ca^{++}$ influx. Passive permeability to Na^{+} and K^{+} as well as the sodium pump were enhanced. Administration of porcine calcitonine largely prevented these modifications, as well as the development of the athero- arteriosclerotic plaques (Jacob et al. 1987b). Treatment with calcitonin largely prevented also the calcification of elastin fibers in these animals (Jacob et al. 1987b). The inhibition of Ca-influx by calcitonine might well be the key factor in these experiments.

Among the not completely elucidated consequences of the above summarized immun-mechanisms remain the effect of possible age-dependent modifications of immun-functions on elastin induced athero-arteriosclerosis. Age-dependent modifications of the human and animal immun-systems were extensively investigated over the second half of the 20th century (Robert L, Robert B. 1973 for a review). The described age-dependent modifications of the immun-systems might well influence the outcome of the immun-atherosclerotic process also. As however the emphasis of our research and the interest of other teams shifted to the age-dependent modifications of receptor function (Joseph and Roth, 1990; Robert L. 1998 for reviews; Roth 1979) the interpretation of the observed pathological modifications shifted progressively towards postgenetic (epignetic, posttranscriptional) mechanisms (Robert L., Labat-Robert J., 2000 for review). Several experimental facts pleaded in favor of the progressive preponderance of such mechanisms. Although the selective uptake of cholesterol by the vascular elastin fibers were first demonstrated in vivo in animals (Jacotot et al. 1973, Robert L. et al. 1984b; Szigeti M. et al. 1972) qualitative and quantitative determinations of lipid classes strongly associated with purified human elastin carried out on human aorta samples (Claire et al. 1976) confirmed the same strong affinity of human elastin also to lipids, attributed to factors inherent to the hydrophobic nature of elastin. Circulating elastin peptides present in all human sera examined were shown to exhibit a number of relevant pharmacological properties (Robert L. et al. 1984a) important also for lipid mediated processes during atherogenesis.

Some remarks on circulating elastase-type endopeptidases. Their upregulation could be, at least partially attributed to circulating elastin peptides as described in previous sections of this review. The presence of identical or very similar peptide sequences in elastin of several species might also contribute to the explanation of the incomplete elimination of elastin-recognizing immunocompetent cells during human development. And finally the demonstration of increased elastin mobilisa-

tion with cholesterol feeding alone (Jacob et al. 1982, 1983) might have justified to some extent the neglection of the immune-mediated mechanisms in human atherogenesis. With the rapid increase of human longevity this approach might however reach its limits, producing a revival in the interest of immune-mediated processes in human vascular diseases.

5 Conclusions

The above summarized experiments and conceptions support the contention for an immuno-inflammatory mechanism involved in the development of athero-arteriosclerosis. Although most studies were conducted on animal models, the findings on humans reviewed in the preceeding sections strongly support the validity of the elastin-antielastin antbody-based process as of importance also for human pathology (Robert L. et al. 1967, 1968, 1970b, 1984c; Robert B., Robert L., 1974c, 1975). The connection of this process to the ER mediated pathways via elastin degradation and elastin peptide liberation in the circulation is of importance for the rationalisation of the role for both immun-mediated and receptor mediated mechanisms. There is also the connection to the lipid-mediated processes, essentially by the upregulation of elastase production by the atherogenic lipoproteins, increasing therefore the local liberation of elastin peptides. Not all elastin peptides are however equivalent in this respect. Synthetic hexapeptides made on the pattern of several exons of the elastin-gene were used for screening with monoclonal antibodies (Wei et al. 1993) and for triggering the ER on endothelial cells (Faury et al. 1998b). These and other studies revealed the sequences recognized by the ER, corresponding to the pattern GXXPG comprising the most studied peptide, VGVAPG. Using the endothelial cell model it could be shown however that even tripeptides as VGV can already trigger the ER (Faury et al. 1998b). Although all the above cited studies enlarged the scope of the initially proposed immune-theory of atherogenesis, its essential features remained valid, close to the original hypothesis (Robert L. et al. 1967, 1968). Further experiments are clearly needed essentially to explore the relevance of notions acquired during the study of the ER and their physiopathological consequences as well as their connection to known risk factors of atherogenesis. It seems probable that such studies could open new vistas for original pharmacological innovations.

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Cancer

Aging, Immunity and Cancer

Claude Sportès and Frances T. Hakim

Contents

Abbreviations

ADM	AIDS defining malignancies
AT	Ataxia-Telangiectasia
EBV	Epstein Barr virus
FA	Fanconi's Anemia
FasL	Fas Ligand
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GVT	Graft-versus-tumor
INF-γ	Gamma interferon
IL-1β / 6	Interleukin-1β / 6
MCA	Methylcholantrene
nADM	non-AIDS defining malignancies
NFκB	Nuclear Factor-κB
RAG-2	Recombinase Activating Gene -2
S.E.E.R.	Surveillance, Epidemiology and End Results
TNFα	Tumor Necrosis Factor-α
XLA	X-linked Agammaglobulinemia

Abstract: Although the increased incidence of common cancers with age on one hand and immunosenescence on the other are both well documented in animal

C. Sportès (✉) · T. Hakim
Experimental Transplantation & Immunology Branch
Center for Cancer Research, National Cancer Institute
10 Center Drive, Bldg 10-CRC Room 43142
Bethesda, MD 20892-1203
Tel: (301) 435 52 80
Fax: (301) 402 75 15
E-mail: csportes@mail.nih.gov

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models and in humans, evidence for a causal link between the two is controversial. It is, therefore, most appropriate to critically consider whether the dysfunctional immune processes occurring with aging may play a contributing role in the increased incidence of cancer with aging. Indeed whether or not immunosenescence plays a significant role could have a determinant impact on devising cancer prevention and/or immunotherapeutic strategies for the presently growing elderly population.

Keywords: Immuno-surveillance • Primary immuno-deficiency • Adoptive immunotherapy • Inflammation • Immuno-editing

1 Introduction

Aging involves most if not all physiologic systems. As knowledge about the aging process increases, it becomes apparent that it cannot be viewed simplistically as the decay phenomenon of the various parts of a complex machine but, rather, as the development of active processes that are maladapted or dysfunctional and actively lead to progressive organ or system dysfunction. Some of these processes may be initially triggered by a phenomenon of aging but the clinical consequences of this initial phenomenon may be considerably amplified by these active processes which, therefore, should become the primary target of anti-aging therapeutic or prophylactic interventions. The understanding of the pathophysiology of these processes may lead to the discovery of effective anti-aging interventions since they are more likely to be amenable to effective therapeutic interventions than primary phenomena of aging. Immuno-senescence, the aging of the immune system, to which this entire volume is dedicated, is no exception to this approach to understanding the overall physiology of aging.

Although the increased incidence of cancer with aging is a fact, such a general statement does not accurately represent the complexity of the phenomenon and, therefore, may lead to erroneous interpretation. In this chapter we will review the multiple lines of evidence that the immune system has an active role in detecting and eradicating nascent tumors grouped under the broad concept of immuno-surveillance. This concept is widely accepted and based mostly on the presented evidence in various clinical and experimental immuno-deficient states. There is, therefore, an obvious connection to be made between the progressive state of immune deficiency called immuno-senescence, a putative lack of adequate immune-surveillance (in analogy to the severe immune deficient states) and the increased incidence of cancer during aging. Another connection to be easily made is between the accumulating knowledge that chronic inflammation is a hallmark of many of the critical aging processes and the developing evidence that growing tumors may recruit normal inflammatory processes for their own benefit, protection and growth. Finally, the concept of tumor immuno-editing is an attempt to consider more dynamically the interplay between the multiple opposing forces implicated in tumor development and tumor eradication. This concept offer a platform to critically discuss

the hypothesis that immuno-senescence contributes significantly to the increased incidence of cancer with aging.

2 Evidence for Increased Incidence of Cancer with Aging

The increased incidence of the most common cancers with age is well established and will be only briefly summarized. The lifetime risk of cancer is about 1 in 2 in men and 1 in 3 in women. Cancer incidence shows a steep increase after the age of 65, but plateaus for most common cancers between age 80 and 85 and even decreases thereafter. It should be noted, however, that this global increase in incidence of cancer is predominantly caused by the peak incidence of the most common malignancies (prostate, lung, colo-rectal, breast, bladder, and pancreatic cancers) in the later decades of life. It is interesting to note that, for most of these common cancer types (except colo-rectal and bladder cancers), incidences both for males and females start to decline after age 80 to 85. Table 1 shows recent data obtained from the Surveillance, Epidemiology and End Results (S.E.E.R.) database on the overall incidence over age 65, the peak incidence and age and the age after which the incidence declines. The late decrease in incidence may be the simple reflection of the bell shaped curve distribution of new cancer cases, centered on the peak incidence. Hence, it could be convincingly argued that the observed global increased incidence of cancer in older age is, in fact for the most part, an artifact (Zhang and Grizzle 2003) and does not specifically result from patho-physiologic mechanisms found uniquely in the aging population (such as

Table 1 Incidence, peak incidence and declining incidence of most common cancers

Tumor type	Incidence over age 65		Peak incidence		Age of Peak incidence		Declining incidence (after age.)	
	Male	Female	Male	Female	Male	Female	Male	Female
Prostate	924.6	-	1,021	-	70-74	-	+ (80)	-
Lung	469.5	282.5	570.2	336.1	75-79	75-79	+ (80)	+ (80)
Colon	334.3	243.8	460.5	377.4	85+	85+	No	No
Breast	-	424.4	-	464.8	-	75-79	-	+ (80)
Bladder	228.1	54.1	330	79.6	85+	85+	No	No
Pancreas	71.4	58.9	93	87.7	80-84	85+	+ (85+)	No
Melanoma	96.9	38.2	119.7	42.7	80-85	80-85	+ (85+)	+ (85+)
Corpus Uteri	-	83.5	-	89.1	-	65-70	-	+ (70)
Kidney	78	37.9	88.1	43.1	75-79	75-79	+ (80)	+ (80)
Ovary	-	49.8	-	58.1	-	80-84	-	+ (85+)
Brain	23.9	15.7	28.3	18.7	75-79	75-79	+ (80)	+ (80)
All cancer sites	2875.3	1686.5	3266.4	1968.1	75-79	80-84	+ (80)	+ (85+)

Data obtained from the S.E.E.R. database (http://seer.cancer.gov/csr/1975_2004/sections.html)
Incidence given per 100,000 individuals in population.

immunosenescence). In particular, one can argue that, if immuno-senescence were a significant factor in the phenomenon of aging and increased cancer incidence, one would expect to see a continued rise in cancer incidence with age in the 9th and 10th decades of life, if immuno-senescence continues to progress.

The increased cancer incidence with increasing age underlies complex mechanisms, linked to various degrees to a multiplicity of active processes associated with aging and, therefore, it may be considered a consequence of aging. Alternatively, it is also clear that the progressive accumulation of either genetic alterations or other cellular insults resulting in malignant transformation is time dependent and that, therefore, cells in older individuals will be more likely to accumulate a critical number of insults necessary to undergo such transformation. In that sense, the increased incidence of common cancers later in life is not due to aging but to being older.

3 Evidence for Tumor Immunosurveillance

There is ample evidence for a critical role of the immune system in the defense against various cancers before any of the aging processes become clinically significant which defines immunosurveillance and immuno-editing of cancer.

3.1 Immunosurveillance in Animal Models

After being first proposed in the early 20th century, the concept of tumor immune surveillance fell in disfavor after initial experiments failed to demonstrate the proof of principle that immuno-deficient (athymic nude) mice would have an increased incidence of cancer when compared to their immuno-competent heterozygote littermates (Stutman 1974). As monoclonal antibody and transgenic technologies developed, allowing systematic targeting of specific immune pathways, proof of principle was demonstrated and spawned a renewed interest in the concept of tumor immuno-surveillance.

A large number of animal models have been developed, generating immuno-deficient mice with enhanced sensitivity to chemically induced and spontaneous tumors. Some of these models are also informative on the increased incidence of cancer with aging. The state of knowledge of immuno-surveillance has been the object of several recent reviews (Dunn et al. 2004; Dunn et al. 2006; Swann and Smyth 2007). Multiple mouse knockout experiments have been performed on various mouse genetic backgrounds with varying results underscoring the influence of the genetic background in the nature and efficiency of immune surveillance for a given gene defect (Street et al. 2002). Some models evaluated the spontaneous occurrence of tumors while others used tumor induction with a carcinogen (Shankaran et al. 2001) or an added transgenic defect known to induce tumors (e.g., double knockout $p53^{-/-}$ / pore forming protein $^{-/-}$ or INF-γ insensitivity [Kaplan et al. 1998; Smyth et al. 2000]).

Two major systems have been described to be involved in immune mediated tumor suppression based on these mouse knockout models. *The gamma interferon (IFN-γ) pathway*: mice insensitive to INF-γ (129/SvEv mice) lack either the INF-γ receptor (INFGR1) or STAT1, a critical transcription factor in mediating INF-γ receptor signaling. These mice are 10-20 times more sensitive to tumor induction with methylcholantrene (MCA) than their wild-type counterparts (Kaplan et al. 1998). *The perforin pathway*: Perforins are critical in the cytotoxicity of T-cells and NK-cells. Perforin-null mice show increase spontaneous tumor formation (lymphomas or epithelial tumors [Street et al. 2002]) as well as increased MCA-induced tumors. The RAG-2 (Recombinase Activating Gene) knockout experiments also brought a higher degree of specificity to the immune mediated tumor suppressor model since this gene is exclusively expressed in the immune cells (T-, B- and NKT-cells) at the time of their antigen receptor gene rearrangements. Therefore, RAG-2 deficient animals have no T-, B- or NKT-cells. In these experiments, 60% of the RAG-2 deficient mice versus 20% of the wild-type mice developed tumors (Shankaran et al. 2001). In older animals (age 13 to 24 months) not exposed to MCA, 100% of the RAG-2 deficient animals developed spontaneous tumors, most being malignant versus 25% of the wild type animals, mostly developing benign tumors. Interestingly, in this system, the double knockout mice ($RAG2^{-/-}$ / $STAT1^{-/-}$) did not show an increase in tumor generation over either single knockout strain, suggesting that the protective effects of the INF-γ pathway may be mediated through the adaptive immune system.

3.2 Immunosurveillance in Human Immunodeficiencies

The evidence for an operational immune surveillance system in humans is not as scientifically compelling as in these precisely defined animal models but, yet, is quite convincing. It stems from two distinct groups of immune deficient individuals: subjects with congenital primary immuno-deficiencies and subjects who develop immune deficiency later in life, such as from HIV infection or immunosuppressive therapy in the context of organ transplantation.

3.2.1 Primary Immunodeficiencies and Cancer

As is so often the case, valuable insight in broad patho-physiologic concepts can be gained from observation in very rare diseases. The evidence for a significantly increased incidence of predominantly lymphoid malignancies in primary immunodeficiency diseases is indisputable (Ochs et al. 2006). This includes lymphoid malignancies that are either virally associated or not but the most consistent finding in these congenital immuno-deficiencies is that a disproportionate number of the tumors that develop are virally associated, if not virally-induced. This emphasizes the major role of the adaptive immune system in the long term control and eradica-

tion of viral infections and the causal link, direct or not, between viral infections and many malignancies: EBV and Hodgkin's lymphoma or EBV—lymphoproliferative disease, Hepatitis B and C viruses and Hepatocellular carcinoma, human Papilloma virus and cervical cancer, human Herpes virus 8 and Kaposi sarcoma.

Interestingly, of all the most common primary immunodeficiency diseases, X-linked Agammaglobulinemia (XLA) is the only one that does not seem to be associated with an increase incidence in lymphoid malignancies (Table 2). This suggests that antibody mediated immunity has little role in immune surveillance of cancer in humans. Data on the incidence of nonlymphoid and nonvirally associated malignancies in these rare diseases are, however, much less compelling.

Table 2 Primary immunodeficiency diseases and cancer susceptibility Compiled from [Ochs et al. 2006]

Syndrome	Other Defect	Increased Lymphoid malignancies	Increased Nonlymphoid malignancies
Ataxia Telangiectasia (AT) [Varan et al. 2004]	yes	yes	yes
Bloom Syndrome	yes	yes	yes
Cartilage-Hair Hypoplasia (CHH)	possible	yes	not clear(1)
Fanconi's anemia (FA)	yes	yes	yes
Nijmegen Syndrome	yes	yes	Possible(2)
Autoimmune lymphoproliferation sd (ALPS)	no	yes	not clear(3)
CD40 L deficiency (Hyper IgM syndrome)	no	yes	not clear(4)
Common Variable Immunodeficiency (CVID)	no	yes	Gastric ca(5)
Hyper IgE syndrome (Job syndrome)	no	yes	no
Wiskott Aldrich Syndrome (WAS)[Sullivan et al. 1994]	no	yes	not clear
X-linked Agammaglobulinemia (XLA)	no	not clear	no

(1) In adulthood, 90-fold increase in lymphoma NHL over general population and 6.9-fold for other cancers. The function of deficient gene is unknown (leaving open the speculation on other pathogenesis of cancer)
(2) 40% of patients develop malignancy before age 21. The overwhelming majority are leukemias / lymphomas. Second malignancies are lymphomas with much rarer instances of medulloblastoma, rhabdomyosarcomas, Gonadoblastoma and Ewing sarcoma. The defective gene may function as a tumor suppressor gene.
(3) Hepato-cellular Ca (Hepatitis C +) at age 43, Basal cell Carcinoma. The frequency is not clearly distinct from that in general population.
(4) Liver and biliary tumors may be linked to increased incidence of local infections
(5) 50- to 100-fold increase in lymphomas. Gastric carcinoma may be linked to deficient immunity to H. pylori.

In all the syndromes that show either a definite or a possible increased incidence in nonlymphoid malignancy (Table 2), there is either clear or suggestive evidence that other carcinogenic mechanisms are most likely responsible for the increase (e.g., chromosomal instability in Fanconi's Anemia (FA), Ataxia-Telangiectasia (AT), Bloom syndrome, etc). Conversely, among the congenital immuno-deficiency syndromes that do not seem to involve either chromosome instability or other known cancer susceptibility mechanisms (e.g., XLA, Wiskott Aldrich Syndrome, CD40L deficiency), it is either difficult to find a definite increase in incidence of non-lymphoid tumors over the general population or the increased incidence is more likely due to chronic infection and/or inflammation secondary to the immunodeficiency state than to a defective tumor immuno-surveillance per se (Hayward et al. 1997). It is however noteworthy that the putative increase in non-lymphoid malignancies appears to remain mostly in "age appropriate" tumors unless a mechanism of carcinogenesis other than immune deficiency can be incriminated (e.g., FA and epithelial tumors). This latter fact is concordant with the concept of active, ongoing tumor immune surveillance because the few mechanisms of carcinogenesis that are operational in this early age group, if not well controlled by an efficient immune surveillance system, would be expected to result mostly in an increase in age appropriate tumors rather than in adult type tumors.

As the overall incidence of cancer (and in particular of epithelial cancers) is considerably less in children than in adults owing to differences in carcinogenic mechanisms in the two populations, it remains difficult to extrapolate the data from congenital immuno-deficiencies to the adult and aging population. Therefore, one must seek evidence in immuno-suppressed adult populations.

3.2.2 Cancer and the Immuno-suppressed Host

There is an abundant clinical literature on the increased incidence of tumors in individuals treated with various immuno-suppressive therapies for solid organ transplantation. Initially, tumors developing in the context of immuno-suppression were believed to be mostly virally related (EBV lymphoproliferative disease, Kaposi sarcoma) or lymphoproliferative diseases. This was felt to be consistent with what is seen in the congenital immuno-deficiency syndromes described above. More recent studies of large cohorts of organ transplant recipients, however, show a significant increased risk in the more common tumors such as lung, prostate, colon carcinomas as well as melanomas and non melanoma skin cancers and nonvirally induced sarcomas (Penn 1995; Penn 1996; Pham et al. 1995). The state of knowledge on malignancy in transplantation has been recently reviewed (Buell et al. 2005).

With the recent advent of effective antiviral and other antiinfectious therapies which have allowed prolonged survival of HIV infected individuals, much information about the role of immune surveillance in the development of cancer is now also being accumulated in this population. It is becoming increasingly clear that the higher incidence of malignancy in HIV disease is seen in both AIDS defining

malignancies (ADM: Kaposi sarcoma, non-Hodgkin lymphoma and cervical carcinoma) and non-AIDS defining malignancies (nADM). Data from the prospective, multinational Data Collection on Adverse Events of Anti-HIV Drugs study (DAD) was recently presented on follow up of 23,437 HIV infected individuals (for a total of 104,961 patient-years follow up) (D'Arminio Monforte et al. 2007). Only 37% of all deaths from malignancy were due to ADM. This study demonstrated that a low CD4 T-cell count and not HIV RNA copy number was correlated with the increased incidence of nADM. Twenty percent of all deaths from malignancy were due to lung cancer, the most commonly occurring nADM and, although smoking remained the greatest risk factor for the increased incidence of lung cancer in this HIV population, low CD4 T-cell counts were significantly correlated with the incidence of lung cancer, independent of the smoking history. These data suggest first, that the increase incidence of lung cancer in the HIV population is not entirely due to the high prevalence of smoking in this population and second, that the consequence of HIV infection (i.e., immuno-suppression with low CD4 T-cell counts), rather than a direct viral effect of HIV, is responsible for the increase incidence of lung cancer.

3.3 Immunosurveillance in the General Immune Competent Population

There is evidence in the general immune competent population that the degree of host immune response is correlated to cancer prognosis. In an 11-year follow-up longitudinal study of a general population, higher natural cytotoxic activity of peripheral-blood lymphocytes was found to be correlated with a decreased cancer incidence (Imai et al. 2000) and circulating natural killer cell activity has been found to be of prognostic significance in patients with gastric carcinoma in a multivariate analysis (Takeuchi et al. 2001). This study was the first prospective cohort study to link, in a normal population, host immune defenses (in this case innate immunity) and the incidence and prognosis of cancer. It is also noteworthy that, to date, no comparable epidemiologic study has linked similarly adaptive immunity and cancer. However, there is also now growing clinical evidence that the presence of tumor infiltrating lymphocytes is associated with a better survival in a variety of tumors including ovarian cancer (Zhang et al. 2003), colo-rectal cancer (Chiba et al. 2004; Pages et al. 2005) and others (Abe et al. 2003; Cho et al. 2003; Hiraoka et al. 2006; Nakakubo et al. 2003; Wakabayashi et al. 2003).

3.4 Clinical Evidence for Immune Eradication of Tumors

Demonstrating the proof of principle of successful active cancer immunotherapy, immune mediated eradication of clinically relevant tumors in humans can be spectacular, albeit documented thus far only in a small number of cases. High-dose interleukin-2 in the treatment of metastatic melanoma or renal cell carcinoma results in durable clinical remissions in a significant minority of patients (Atkins et al. 1999;

Phan et al. 2001; Rosenberg 2000). Intensive research on various forms of adoptive cancer immunotherapy with autologous lymphocytes infusion has been pursued for many years with some definite but unfortunately still limited success. Recently, the induction of a profound immuno-depletion prior to the reinfusion of expanded tumor infiltrating autologous lymphocytes has led to significant and durable tumor responses in metastatic melanoma. These studies and parallel murine studies have led to new hypotheses for the mechanisms at play in effective immunotherapy (Paulos et al. 2007). Encouraging results such as these extend cancer immunotherapy beyond the stage of proof of principle (Dudley et al. 2005; Gattinoni et al. 2006).

Some tumors developing in severely immuno-suppressed individuals following solid organ transplantation can be completely eradicated with a simple withdrawal of their immuno-suppressive therapy (Wilson et al. 1968). Furthermore, following allogeneic stem cell transplantation, individuals (mostly with recurrent chronic myelogenous leukemia but also with other diagnoses) can be re-induced into a durable complete molecular remission simply with infusion of lymphocytes from their transplant donor (Collins et al. 1997; Mackinnon et al. 1995a; Porter et al. 1994; Porter et al. 1997). These results, along with the evidence of the poor tumor control following T-cell depleted allogeneic stem cell transplantation or stem cell transplantation from identical twins, has led to the realization that the immunologic graft-versus-tumor (GVT) effect may be, in certain diseases, as important as the high dose radio-chemotherapy in the overall therapeutic effect of allogeneic hematopoietic stem cell transplantation. This has led to the development of the whole new field of clinical research in non-myeloablative stem cell transplantation which attempts to maximize the alloreactive GVT effect and minimize toxicity from the radio-chemotherapy preparative regimen (Khouri et al. 1998). Interestingly, the donor lymphocyte dose needed to eradicate the primary disease occurring spontaneously prior to the recipient being immuno-suppressed (e.g., relapsed chronic myelogenous leukemia) is greater than the lymphocyte dose needed to eradicate a secondary, virally induced malignancy (EBV lymphoproliferative disease) that occurs during the immuno-suppression period of the transplant (Mackinnon et al. 1995b). An alloreactive GVT effect has also been demonstrated for some solid tumors such as breast and renal cell carcinoma (Bishop et al. 2004; Childs et al. 2000; Ueno et al. 2003). These latter two observations are very interesting since they indicate that some tumors that develop in normal, non immuno-suppressed individuals (and therefore have become non-immunogenic to the host) remain immunogenic to the donor T-cells in the context of the alloreactivity created by the transplant and that second malignant tumors developing in the context of host immuno-suppression remain more immunogenic than the primary tumor.

4 Role of Chronic Inflammation in Cancer and in Aging

Acute inflammation occurs in response to invasion by infectious agents or tissue injury. It is the first line of defense of the body and is operated by the innate immune system. This initial defense response is intimately linked to the subsequent development of an efficient response by the adaptive immune system. When this initial

inflammatory response results in the eradication of the infectious agent or in healing of the tissue injury, the inflammatory process ceases. Persistent, chronic inflammation, however, may have many deleterious effects. Many causes of chronic inflammation unrelated to aging are linked to the development of specific malignancies and, conversely, human epidemiologic studies and murine experimental models have established the protective role of chronic anti-inflammatory drugs (aspirin, COX-2 inhibitors) against the development of cancer such as colon or breast cancers (Steinbach et al. 2000). The role of an inflammatory microenvironment and tumor development has been recently reviewed (de Visser and Coussens 2006).

Cancer promoting inflammation can be perpetuated by a specific disease process (e.g., ulcerative colitis and colon carcinoma [Eaden et al. 2001] or chronic pancreatitis and pancreatic cancer [Whitcomb 2004]), by the incomplete eradication of an infection (e.g., Hepatitis B or C and hepato-cellular carcinoma [Donato et al. 1998]) or by a chronically recurrent infection (e.g., Helicobacter pylori and gastric carcinoma [Ernst and Gold 2000]), as well as by repeated low level tissue injury caused by prolonged exposure to chemical (e.g., chronic gastro-esophageal reflux and esophageal carcinoma [Cameron et al. 1995]) or physical (e.g., asbestos and mesothelioma [Manning et al. 2002] irritation). In many of these associations, the clinical data are corroborated by animal models of specific tumor induction following repeated exposure to the specific offending agent that leads to the chronic inflammation. In some of these animal models, B-cells have been shown to be critical, linking innate and adaptive immune responses in inflammation induced carcinogenesis (de Visser et al. 2005). Exaggerated responses to pro-inflammatory stimuli such as LPS (e.g., increased Tumor Necrosis Factor-α (TNFα), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6)) may promote tumor through the Nuclear Factor (NF)-κB / IKK pathway (Karin and Greten 2005; Lin and Karin 2007) and the role of the activation of the NFκB / IKK pathway provides a mechanistic link between inflammation and carcinogenesis (Karin 2006). Some mouse models of de novo carcinogenesis also point to the critical involvement of an inflammatory process in the carcinogenesis. GM-CSF$^{-/-}$, IFN-γ $^{-/-}$ double knockout mice develop spontaneous lymphomas and solid tumors on a backdrop of persistent inflammation and recurrent infections. These mice can be protected from developing tumors by an aggressive antibiotic therapy from birth, decreasing the bacterial load (Enzler et al. 2003).

Chronic inflammation is not only involved in the pathogenesis of tumors, it also plays a significant active role in tumor progression, aggressiveness and metastatic potential. Although in situ tumor infiltration with cells of the adaptive immune system is often associated with improved survival and better tumor control (Abe et al. 2003; Chiba et al. 2004; Cho et al. 2003; Hiraoka et al. 2006; Nakakubo et al. 2003; Pages et al. 2005; Wakabayashi et al. 2003; Zhang et al. 2003), the presence of cellular infiltrates of the innate immune system (mostly macrophages and myeloid cells) is often associated with more aggressive tumor progression (Lin and Pollard 2004a; Lin and Pollard 2004b). The understanding of the interactions between tumor-associated macrophages and the tumor is the object of particularly intensive investigations that may lead to novel therapeutic approaches (Pollard 2004).

The dichotomy of beneficial effects of the adaptive immune system opposed to deleterious effects of the innate immune system is, however, an oversimplification since circulating NK activity has been found to be of good prognostic significance in patients (Takeuchi et al. 2001) while B-cells and regulatory T-cells have been implicated in the development of an immunosuppressive milieu in the tumor microenvironment which favors tumor progression. The state of knowledge of these complex interactions has been recently reviewed (Bronte et al. 2006).

A critical balance between pro-inflammatory and anti-inflammatory responses is maintained at all times. It involves complex local responses but also multiple systemic responses including the nervous system (Tracey 2002). On one hand, an insufficiency in pro-inflammatory signals may lead to an increase in numbers of infections or cancers due to decreased immune surveillance. On the other hand, an insufficiency of anti-inflammatory signals may result in increased morbidity or mortality due to exaggerated proinflammatory responses (e.g., shock in response to infection) or in increased autoimmunity, atherosclerosis and cancer. There is indeed considerable evidence for a shift toward a proinflammatory state during aging and for chronic inflammation playing a critical role in the pathogenesis of most of the prevailing diseases associated with aging including Alzheimer disease, Type II diabetes, atherosclerosis, osteoporosis, arthritis and wasting syndrome. The multitude of elements (cytokines, chemokines, cellular components) at play in maintaining the inflammatory balance, the alterations of these various elements leading to chronic systemic low level inflammation with aging and the clinical impact in the diseases of the elderly have been recently reviewed (Bruunsgaard 2006) and are specifically addressed in other chapters of these volume.

5 Cancer and Immuno-senescence

Although the increased incidence of cancer with age on one hand and immunosenescence on the other are both well documented in animal models and in humans, the evidence for a causal link between the 2 is controversial. It is generally accepted that histologically similar tumors grow more slowly, with less angiogenesis, in aged mice relative to young mice (Reed et al. 2007). This is in keeping with the human experience.

5.1 Concept of Immuno-editing Deficiency

Recently, a concept more global than immuno-surveillance, called immuno-editing, has emerged in order to include the on-going interactions between a developing tumor and its destruction by the immune surveillance system (Dunn et al. 2004).

A critical aspect of the experiments in various immune deficiency models described above in section 3.1 is the demonstration that tumor development is in large part the

consequence of the failure of, or the escape from, the immuno-surveillance machinery and that the intact immuno-surveillance system of an immune competent host actively contributes to the selection of poorly immunogenic tumors. Indeed, tumors that develop in immune deficient mice, either spontaneously or following exposure to a carcinogen can be very efficiently rejected when injected into the wild-type immune competent counterpart strain, demonstrating that these tumors remain immunogenic. However, tumors induced by the same carcinogen in a wild-type immune competent animal cannot be rejected by syngeneic animals, suggesting that the intact immune surveillance system of the wild-type animals progressively eliminated the immunogenic components of the developing tumor resulting in the selection of poorly immunogenic tumors. Meanwhile, the tumors developing in the immune deficient mice, having not been subjected to immune pressure, remained immunogenic for competent hosts (Engel et al. 1997; Shankaran et al. 2001). The cause of the immuno-deficiency (e.g., IFN-γ or perforin deficiency), operating on various genetic backgrounds, and its possible association with other genetic defects may also lead to varying manifestations of tumor immuno-selection leading to different immunogenicities of the resulting tumors (Street et al. 2002).

The constant emergence of mutant tumor cells (due to their inherent genetic instability and interactions with their micro-environment) repeatedly challenges the system of immune surveillance to adapt and develop new ways to eradicate the newcomers. This on-going interplay takes place in the first two phases of the cancer immunoediting: elimination and equilibrium. These processes therefore contribute to what has been coined the "immunologic sculpting" of a tumor during its development, the immunogenic components of the tumor being progressively eliminated (Dunn et al. 2004) by the immune surveillance mechanisms.

The third phase (escape) begins when a nascent, sub-clinical tumor develops characteristics that either render it insensitive to the immune surveillance or blunt the immune surveillance responses (e.g., tolerance induction), thereby allowing its development into a clinically relevant tumor. This model predicts that tumors developing in immune deficient animals would remain more immunogenic than the ones developing in immune competent animals, as their survival did not require refining an ability to elude a fully functional immune system. This prediction is also confirmed in the previously mentioned human clinical experience in the immuno-suppressed host where tumor eradication is achieved by simple withdrawal of immuno-suppressive therapy, indicating that tumors developing in immunocompromised human hosts tend to remain more immunogenic, presumably by lack of effective immuno-editing (Mackinnon et al. 1995b).

The concept of immunologic sculpting of nascent tumors also predicts that the nature of the immune pressure delivered by an aging immune system to arising tumors may evolve with time resulting in a different tumor evolution without being considered to be the result of an immune deficiency per se but simply because the tumor is submitted to a different immune pressure. For example, studies have demonstrated an increase in Fas receptor (CD95) expression with age, either as percentage of Fas-expressing T-cells or as mean fluorescence intensity per T-cell, concomitant with the increased frequency of memory and effector T-cells. This increase in

Fas receptor expression might facilitate the immune escape of Fas ligand (Fas-L) expressing tumors in elderly patients by promoting tolerance via the apoptosis of tumor specific leukocytes. In the sense that tumor editing by a senescent immune system will result in somewhat different tumors than in younger individuals, immuno-senescence can be considered to have an impact on tumor generation in the elderly. The question to answer in humans would be: are tumors arising in older individuals more immunogenic than in younger individuals? In keeping with the experimental data mentioned above, this would argue in favor of immuno-senescence specifically allowing a greater immune escape for tumors. Such a question cannot be answered directly in humans. The answer would have to come from large epidemiologic studies or the realization, in future clinical trials, that effective cancer immunotherapy (unfortunately, yet to be developed) is more effective in older than in younger individuals although this would only be indirect evidence.

The immuno-editing model presents arguments both for and against immuno-senescence being a cause for the increased incidence of cancer in the elderly. On one hand, it may be argued that the repeated assaults of mutating tumor cells on the immune surveillance system are more likely to be successful as the immune system weakens, be it due to pathologic (HIV or primary immuno-deficiencies), iatrogenic (immuno-suppressive therapies) or physiologic (age) causes. On the other hand, the model also implies that the longer tumor cells are present the more likely they are to ultimately find the Achilles' heel of immune surveillance, regardless of its strength. Overall exposure to mutating tumor cells will inevitably increase with age and, therefore, immune escape would be expected to become more prominent with time.

5.2 Animal Model Arguments

A very dramatic model of heightened cancer immuno-surveillance is found in the SR/CR strain of mice (Cui and Willingham 2004) which developed a still poorly elucidated genetic defect. This strain displays a very powerful resistance to a variety of highly lethal mouse tumors. Eradication of transplanted tumors occurs with a massive tumor infiltration with cells from the innate immune system. This appears to be the result of a highly coordinated response since the depletion of single leukocyte populations does not abrogate tumor protection. Interestingly, this genetically determined trait is highly age dependent as the mice lose their tumor protection ability between age 6 and 12 months (Cui et al. 2003). Leukocytes of young SR/CR mice lose their tumor protection functional ability when transferred to normal older mice but not when transferred to normal younger mice, suggesting that it is the "older environment" that is responsible for the loss of tumor protection while the cells themselves have the ability to remain functional when transferred in the younger animals. Although this model is useful to generate hypotheses of tumor surveillance mechanisms, it does not shed direct light on the human situation in aging.

The suggestive evidence derived from animal models is informative on the possible mechanisms at play; however, it remains subject to the criticism that it is limited

to very defined animal models that may or may not be relevant to human physiology (Cui and Willingham 2004) or involves mostly severely immuno-deficient animals which do not reflect the physiologic aging conditions in the normal human population. In this regard, the evidence presented above for the role of primary or acquired immuno-deficiencies in human cancer incidence also involves subjects who are much more immuno-suppressed than the general aging population and, therefore, do not reflect either the physiologic aging conditions of the human population at large.

However, if one is to accept that there is indeed a more immuno-deficient milieu surrounding tumors developing in the elderly, it may not only be due to intrinsic defects of the aging immune system as suggested above. Alternatively, the responses to tumor derived immuno-suppressive signals may have a more profound impact in the elderly because of characteristics newly developed in the aging immune system. As previously mentioned, Fas receptor expression is increased in aging T-cells which may then become more susceptible to apoptosis in presence of FasL-bearing tumor cells. Tumor cells secretion of immuno-suppressive cytokines such as TGF-β or IL-10 may synergize with an already known heightened production in the elderly and result in an increased tumor-derived immune suppression. Indeed, some animal models would favor such mechanisms. In a study comparing the impact of the presence of lung tumors on the tumor induced immune dysfunction in young versus old mice, no difference was found in the number of either freshly isolated or anti-CD3 stimulated spleen and lymph node CD4+ T-cells in young and old tumor bearing mice versus non-tumor bearing mice (although there was a overall decline in CD4+ T-cells with age, unrelated to the presence of a tumor); however the IFN-γ secretion by these CD4+ T-cells was significantly more decreased by the presence of tumors in the older tumor bearing animals than in the younger tumor bearing animals. Similarly, although neither age nor presence of tumor had an effect on the number of spleen or lymph node CD8+ T-cells, IFN-γ secretion of these CD8+ T cells was also significantly more decreased in the older tumor bearing animals than in the younger tumor bearing animals (Young et al. 2001).

Some animal models point to a decrease in cell mediated immune surveillance as a cause of decreased tumor protection. Older mice immunized with a HER-2 DNA plasmid are not well protected against a tumor challenge compared to younger mice. Although similar antibody production was found, no anti-p185neu specific cytotoxicity was found in lymphocytes from old animals (Provinciali et al. 2003).

In mice, statistically elaborated T-cell subset patterns, characteristic of aging, can be defined and are predictive of the longevity of the animals which includes resistance to spontaneous lymphoma, mammary adenocarcinoma, and fibrosarcoma. These T-cell patterns are associated with disease development and occurrence of these T-cell patterns earlier in the life of the animals predicts earlier occurrence of tumors, suggesting that, in this model, early immuno-senescence (defined as the occurrence earlier in life of the T-cell patterns characteristic of aging) might predispose to early death from cancer (Miller and Chrisp 2002).

Only few animal studies have attempted to approximate the conditions of normal human aging by studying the duration of exposure to a carcinogen in non

immunodeficient mice. In a large study of normal mice exposed to a carcinogen, while the incidence of tumor was dependent on the duration of carcinogen exposure, it was not age dependent for a given exposure duration. The overall increased incidence of tumors with increasing age could be completely accounted for statistically as a result of increased exposure to the carcinogen and no other putative factors (such as immuno-senescence) needed to be incriminated (Peto et al. 1975). Therefore, the duration of exposure to the carcinogen and the duration of the presence of newly mutated tumor cells in the animals more than the strength of the immune surveillance would determine the generation of clinically significant tumors. Although such studies are very instructive and argue against a specific role for immuno-senescence in the increased cancer incidence with aging in a generally healthy population, one must keep in mind that modeling the exposure to a single carcinogen is not adequate to model the complexity of carcinogenesis in the aging human population and that, therefore, these results cannot be fully extrapolated to the human circumstances.

5.3 Clinical Medicine Arguments

Several examples in clinical medicine argue against immuno-senescence having a significant role in the increased incidence of cancers (Zhang and Grizzle 2003). The incidence of most common cancers increases with aging but, as mentioned previously however, for most, the incidence plateaus around age 80 and declines thereafter while immuno-senescence continues to progress in the subsequent decades. In a study of 507 autopsies of elderly individuals, the prevalence of cancer was 35%, 20% and 16% among people aged 75–90 years, 90–99 years, and over 100 years, respectively while the prevalence of metastases was 63%, 32% and 29% in the same age groups (Stanta et al. 1997). Other data on autopsy records confirm these trends (Miyaishi et al. 2000). If immuno-senescence were playing a significant role in decrease tumor immuno-surveillance, one would expect the cancer rates to continue to increase along with the immune deficiency. In fact, one hypothesis is that the decreased incidence of cancer in the oldest old is in part due to an unfavorable tumor environment created by the alterations of the innate immune system in this age group. In addition, multiple, non immune factors involved in aging could certainly contribute to this phenomenon (Bonafe et al. 2002).

Invasive prostate cancer develops 15 to 20 years following precursor lesions such as prostatic intraepithelial neoplasia. The immuno-editing model predicts that the lag time from benign to malignant lesions must be in part the reflection of an effective immune-surveillance at play. If immuno-senescence were contributing significantly to the increased incidence of prostate cancer in older men, one would expect the time to transformation of a precursor lesion into prostate cancer to shorten as the population ages, reflecting a decline in immunosurveillance capacity with immunosenescence as seen in multiple animal models cited above. This is not the case; the lag time from precursor lesions to prostate cancer remains the same with aging.

Furthermore, if one is to accept that immuno-senescence facilitates tumor escape from immuno-surveillance, one should expect these tumors to develop more rapidly (therefore be more aggressive) since they are unhindered by the failing immune system. This is not true in the case of breast cancer where, when matched for tumor grades and types, there is no difference in clinical outcome between younger and older women.

The risk of developing lung cancer is very much a function of the duration of exposure to smoking and of the length of the smoking-free interval following exposure. The lung cancer risk is reduced by 60% after a 10-year smoking-free period and continues to decline thereafter although the population at risk ages. This would argue against immuno-senescence as contributing to cancer incidence for this tumor. Unfortunately, in order to properly evaluate whether or not smoking cessation in the elderly results, because of a putative effect of immunosenescence, in a lesser reduction of lung cancer risk, one would need to study and compare to the general population of smokers a population of older individuals with the same duration of smoking exposure: a very unlikely population of subjects, older than 70 years of age, who started smoking in their 50's and stopped by age 70. Furthermore, smoking is such a powerful risk factor in lung cancer that any significant increase or decrease in smoking exposure or smoking-free interval will be the predominant influences on lung cancer incidence, overpowering any other potentially true, but weaker, etiologic factors. The impact of a putative decrease in immuno-surveillance effectiveness with immunosenescence may be insufficient to counteract the power of the smoking exposure in the general population and difficult to evaluate statistically. Of interest, as mentioned above, low CD4 T-cell counts in HIV infected individuals now appear to be strongly correlated with lung cancer, independently of the smoking history, suggesting that, in this case, the HIV-induced immuno-deficiency is pronounced enough for its effect to be noticeable.

6 Conclusion

The evidence that immuno-senescence markedly contributes to the increased incidence of cancer with aging is not strong. First, the observed increased incidence of cancer with aging is heavily weighted by the more common cancers that happen to have a peak incidence late in life for reasons most likely irrelevant to immune surveillance. Second, although the clinical evidence where human immunodeficiency is associated with an increased incidence in malignancies is convincing, it is restricted to populations of subjects with severe immuno-deficiencies and it may not be appropriate to extrapolate this evidence to the much less immunodeficient normal aging population. Third, the animal models contributing evidence for the causal link between cancer incidence increase and immuno-senescence are also mostly developed in severely immuno-suppressed animals and of the few studies performed in non severely immuno-suppressed animals some argue against a causal link between the two.

Furthermore, the many processes involved in the pathophysiology of cancer evolve in different directions with aging, some leading to an increased susceptibility and some to a decreased susceptibility to cancer. For example, the time factor leading to accumulation

of more cellular carcinogenic injuries and the phenomenon of immuno-senescence would contribute to the overall trend in the direction of an increased incidence while the selection, in the oldest old, of a residual population who has withstood life either without the occurrence of cancer or with the ability to survive it (possibly due to a combination of favorable genetic polymorphisms associated with longevity) contributes to the overall trend in the direction of an decreased incidence and can explain the decreasing incidence in the 9th and 10th decades of life. Therefore the overall cancer incidence trend cannot be interpreted as the result of a single one of these processes.

In order to further address the question of the role of immuno-senescence in overall cancer incidence, more research must be done, both in models of non severely immunosuppressed animals and in large epidemiologic human studies. These studies must use reliable biologic markers (many of which have yet to be defined and / or validated), not only of immunosenescence but also of other significant factors contributing to the overall cancer incidence such as genetic polymorphisms associated with longevity. Although the difficulty and the complexity of the answer underscore the complexity of carcinogenesis, the question of the role of immuno-senescence in overall cancer incidence remains a very important one to answer, not only for the understanding of cancer development in general but also for the development of future therapeutic or prophylactic immune strategies in the growing aged population.

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Breast Cancer and Immunosenescence

Mauro Provinciali, Alessia Donnini, Arianna Smorlesi and Cristina Gatti

Contents

Abstract: Breast cancer is a disease primarily of older women and, since the elderly population is rapidly expanding, so too will the number of breast cancer patients. The increased incidence of breast cancer in elderly people and its lower aggressiveness have been both related to the age-associated changes occurring in the immune system, the so-called immunosenescence. This phenomenon is best characterized by a remodelling of the immune system, which appears early on and progresses throughout a person's life. The immunosenescence may not only impact on the incidence of breast cancer but also on the effectiveness of preventive and therapeutic approaches based on immune system activation. Immune adjuvants as well as anticancer substances which primarily exert a direct action on tumor cells may have an additive effect on immune-based anticancer approaches, thus playing an important role for the enhancement of immune responses in old ages. This review aims to perform a brief analysis of the age-related alterations of the cell populations involved in antitumour immunity and to analyze the main immunological targets of breast cancer, the effectiveness of immune-based prevention and therapy for breast cancer, and the adjuvant or additive approaches to activate an anticancer immune response in aging.

M. Provinciali (✉) · A. Donnini · A. Smorlesi · C. Gatti
Laboratory of Tumour Immunology
INRCA Res. Dept
Via Birarelli 8, 60121 Ancona, Italy
Fax: 071/206791
E-mail: m.provinciali@inrca.it

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1 Biology of Breast Cancer in the Elderly

Breast cancer is the most common malignancy in women with an age related increase in incidence ranging from 1 in 50 at age 50 to 1 in 10 at age 80 (Rao et al. 2007). With the aging of the western population and rising breast cancer incidence with advancing age, the number of women diagnosed with and surviving breast cancer will dramatically increase over the coming decades. A common view holds that older women develop a form of breast cancer that is intrinsically less aggressive (Silliman and Balducci 1998). In fact, though there is a higher frequency of malignant tumours in the aged, many naturally occurring tumours in humans and laboratory animals are less aggressive with advancing age and permit longer host survival (Ershler and Longo 1997; Balducci et al. 1998). Both the growth and the metastatic spread of breast cancer are slower in older than in younger organisms. The less aggressive characteristics of breast cancer in the elderly have been related to the mechanisms that control the tumor growth in older individuals and, in particular, by the immunosenescence (Kaesberg and Ershler 1989). Weak or nonimmunogenic tumours, like spontaneous tumours in humans, may trigger a low immune response which is unable to reject the tumour but, on the contrary, may cause tumour growth enhancement due to the production of nonspecific growth stimulatory factors by immune cells. In old individuals, the induction of a weaker immune response and the consequent reduced production of growth factors may determine less fertile "soil" for tumour cells (Ershler and Longo 1997). In this context, a direct correlation between tumor growth rate and mononuclear cell infiltration has been observed in primary breast cancers leading to the hypothesis that mononuclear cells produced a tumor growth-stimulating cytokine (Kurtz et al. 1990); the degree of mononuclear cell response was inversely related to the age of the patient. On the other hand, the age-related immune alteration may influence the success of preventive or therapeutic interventions based on immune-system activation, raising the possibility to discover and to employ immune adjuvant or additive approaches for the elderly population (Provinciali and Smorlesi 2005). The need of adjuvant or alternative anticancer approaches based on immune system stimulation is particularly raised by the evidence that traditional therapies are often more aggressive in elderly patients, because of the higher risks of treatment related to the comorbidity associated to old age that renders the elderly a very "frail" patient.

2 Characteristics of Immunosenescence

Experimental and clinical data have demonstrated that ageing is associated with immune system dysregulation, generally characterized with the term of immunosenescence (Solana and Pawelec 1998). Age-associated immune alterations have been

related to the increase of infections, tumours, and autoimmune diseases in the elderly (Thoman and Weigle 1989; Miller 1996; Pawelec and Solana 1997; Burns and Goodwin 1998). For many years, on the evidence that postpuberal thymus involution may be relevant for specific immunity, studies on immunity during ageing have concentrated on the adaptive response and its hallmarks. Many investigators have examined age-related changes in T-cell subsets, in the hope of obtaining clues to the cellular basis of age-associated changes in immune functions. Most of this literature suggests that, in mice and in humans, ageing leads to an increase in the proportion of memory T-cells, and a reciprocal decrease in T-cells with the naïve phenotype, i.e., lymphocytes which have never encountered their specific antigen and that are essential for the induction of primary immune responses against new tumour antigens. The numerical change in lymphocyte representation is accompanied by functional alterations of T-cells that include reduced proliferation, generation of cytolitic effector cells, delayed-type hypersensitivity, and diminished primary and secondary antibody responses (Burns and Goodwin 1998). Furthermore, diminished and/or altered cytokine patterns have been described in old age with lower production of Th1 cytokines, such as IL-2 and IFN-γ and, conversely, higher amounts of Th2 cytokines, such as IL-1, IL-6, IL-8, and IL-10, than young donors (Shearer 1997; Rink et al. 1998). The shift towards a Th2 profile is often associated with a chronic inflammation state in elderly people which, in turn, correlates with processes that contribute to the onset or progression of cancer (Franceschi et al. 2007). Though, the number of circulating B-cells has not been generally reported as changing with age, functional defects of B-cells have been reported in elderly subjects, mainly related to the decline in T-lymphocyte function arising during ageing.

Ageing may affect the antigen presenting cells (APCs) by influencing their antigen processing capacity, the presence of costimulatory signals on their surface, the levels of cytokines in their microenvironment, or their migratory capacity (Provinciali and Smorlesi 2005). It seems that one of the first alterations in APCs in ageing may affect the crucial step of antigen presentation, i.e., the degradation of endogenous proteins, and then the generation of peptides for presentation by MHC Class I molecules. A second defect occurring in APCs, which has been described during ageing is the expression of costimulatory molecules and the regulation of their activity. Though the total number and the expression of MHC I and II, CD80, and CD86 both on immature and mature APCs do not seem to differ significantly in young and old mice (Donnini et al. 2002; Lung et al. 2000), dendritic cells in germinal centres of aged mice were found to lack expression of important costimulatory ligands such as CD86 (Miller et al. 1994), which would promote the induction of anergy in the antigen-specific T-cells with which they interacted. Among the factors that regulate the expression of costimulatory activity on APCs are sets of receptors of the nonclonal innate recognition system called pattern-recognition receptors (PRRs). Of these, toll-like receptors (TLRs) are receptors that recognize conserved molecular patterns, which are shared by large groups of microbial components and are perfectly capable of distinguishing between self and nonself pathogen-associated structures and in turn of signalling the presence of a pathogen to the APCs (Akira et al. 2001). The decreased TLR expression and function recently demonstrated on APCs from aged

mice may have an impact on the antigen presenting function resulting in an impaired immune activation of both innate and adaptive responses (Renshaw et al. 2002). The migratory capacity of DCs has also been found to be affected by the ageing process (Steger et al. 1996a). A lower expression of the mRNA for the migratory CCR7 chemokine receptor was found in APCs from old mice, and a lower lymphocyte cytotoxicity and a reduced number of $CD8^+$ T-cells producing IFN-γ were induced by APCs from aged mice in comparison to APCs from young animals (Donnini et al. 2002). The fact that CCR7 was greatly increased in mature APCs up to the levels found in young animals and that in vivo migration of APCs to regional lymph nodes was higher in old than in young mice, suggests that an increased migratory capacity of old APCs may be required to balance their reduced antigen presentation to cytotoxic lymphocytes (Donnini et al. 2002). The latter assumption is further emphasised by the fact that the lower CTL cytotoxicity induced by APCs from old mice has been attributed to an age-related defect of antigen presentation rather than to an intrinsically lower frequency of cytotoxic T-lymphocytes (CTL). Evidence that the precursors of cytotoxic T-lymphocyte (pCTL) frequency always improved when the source of APCs was changed from old to young animals (Ershler and Longo 1997), and that the transfer of young T-lymphocytes to old mice was unable to correct the deficit in T-lymphocyte responsiveness observed in aged animals (Provinciali et al. 2000) is consistent with this suggestion. Through less evident than in T-cells, age-related alterations have been described also at the level of innate components of cell immunity, such as macrophages, polymorphonuclear leukocytes, as well as natural killer (NK) and γδ T-cells. With regards to macrophages, although early studies conducted in ageing mice or in human subjects showed normal macrophage function (Bar-Eli and Gallily 1979; Jaroslow and Larrick 1973), more recent studies have suggested that macrophage number and function may indeed be altered with ageing. A significant expansion of $CD14^{dim}/CD16^{bright}$ circulating monocytes, which are considered to show phenotypic evidence for activation, has been reported to occur in elderly people (Sadeghi et al. 1999). The constitutive or induced production of IL-1, IL-1 receptor antagonist, and IL-6, was found to increase in monocytes from elderly subjects (Sadeghi et al. 1999; O'Mahony et al. 1998). On the other hand, monocytes from old donors, when compared with monocytes from young subjects, displayed decreased cytotoxicity against tumour cells after LPS activation (McLachlan et al. 1995). Alterations in macrophage number and function have also been described in old rats and mice: impaired TNF-α production, reduced antitumour activity and impaired capacity to produce TNF, IL-1 and nitric oxide, after in vitro activation with IFN-γ and LPS (Wallace et al. 1995; Khare et al. 1996; Corsini et al. 1999), lower expression of MHC Class II gene after incubation with IFN-γ (Herrero et al. 2001), and reduced expression of toll-like receptors (TLRs) on macrophages in ageing mice (Renshaw et al. 2002), were found in either rat or mouse cells. Polymorphonuclear leukocytes (PMNs) have been found among the cell populations more represented in the tumoral infiltrate after in vivo immunization both in young and in old age (Provinciali et al. 2000; Cavallo et al. 1992, 1997). The peri- and intra-tumoral release of cytokines attracts PMNs, as demonstrated by the fact that mice challenged with IL-2-engineered tumour cells were able to reject

the tumour because of direct killing by activated PMNs and macrophages both in young and old age. Several studies show that the neutrophil number in blood and neutrophil precursors in bone marrow, as well as the response to GM-CSF and IL-3, are not lowered in the healthy elderly, albeit, the proliferative response of neutrophil precursor cells to G-CSF was found reduced (Chatta et al. 1993;Born et al. 1995; Angelis et al. 1997). The data from the Literature have reported differences arising from the effects of age on PMN phagocytosis, several studies showing a dramatic decrease in the PMN phagocytic activity from aged individuals (Antonaci et al. 1984; Charpentier et al. 1981). Several groups have examined neutrophil microbicidal activity and, though data are often conflicting, the bulk of evidence supports a decline in cytotoxicity towards bacteria and yeast with age (Corberand et al. 1981; Fulop et al. 1985; Lipschitz et al. 1988).

Whereas an age-related impairment of both endogenous and cytokine-induced NK-cell activities has been commonly reported in mice, the changes occurring in human NK-cell activity with advancing age remain to be fully elucidated. Data from the Literature demonstrates differences in line with the enrolment criteria used to select for the elderly subjects for the study and the use of total lymphocyte populations or purified NK-cells. Overall, both the total and the relative number of circulating NK-cells were found to be significantly increased in healthy elderly people in comparison with young-adult ages. The age-related increase of NK-cell number has been considered as a compensatory mechanism for the decreased cytolytic activity *per cell* found in elderly subjects, (Sansoni et al. 1993). Their direct MHC-unrestricted cytotoxic effects apart, NK-cells have been shown to represent one of the first lines of defense during the early stages of immune activation because of their inducible secretory function. NK-cells synthesize many cytokines and chemokines that can positively or negatively modulate their activity and that of cells of the adaptive immune response. A lower production of IFN-γ, IL-8, and chemokines, was observed in either resting or activated NK-cells taken from healthy elderly subjects in comparison with young subjects (Krishnaraj 1997; Mariani et al. 2001).

Regardless of mechanism, the defect of NK activity in aged mice does not represent an irreversible process, since it may be recovered by hormonal and nutritional treatment (Fabris et al. 1994). Among hormonal factors relevant for NK function, it has been observed that thymic peptides or thyroid hormones, but not the pineal hormone melatonin, were able to restore the crippled NK cytotoxicity of spleen cells from old mice (Fabris and Provinciali 1989; Fabris et al. 1997; Provinciali et al. 1991a, 1997). Among the nutritional factors, either zinc or a lipid mixture which increases membrane fluidity, called “active lipids”, were able to recover the impaired NK function in aged animals (Provinciali et al. 1990a, 1991b). Whether endocrine and nutritional factors have an additive effect or act through the same intracellular mechanism remains to be seen, though the first possibility seems more likely since the action of TSH and thyroid hormones is specifically directed towards lymphokine-boosted NK activity, while active lipids are able to prevent age-associated impairment of basal NK cytotoxicity (Provinciali et al. 1990b, 1991a). Apart from IFN and IL-2, the effect of cytokines on the development of cytotoxic cells during ageing has scarcely been investigated. In a study conducted using IL-12, the

cytokine was able to boost both endogenous and IL-2-induced NK-cell activity in young and old mice. The levels of cytotoxicity were lower in old than in young animals although the relative increase of IL-12 plus IL-2 versus IL-2 alone was greater in old mice (Argentati et al. 2000). These data confirmed and extended previous findings obtained in humans and show that IL-12 is able to enhance NK cytotoxicity to the same degree in both young and elderly subjects, whereas the induction of IL-2-activated cytotoxic cells decreased in elderly compared to young individuals (Kutza and Murasko 1996).

The data from the Literature on numerical or functional changes of γδ T-cells during ageing are scarce and fragmentary. It has been reported that the complexity of the gamma delta T-cell repertoire decreases with age as a consequence of the expansion of a few T-cell clones (Giachino et al. 1994). The analysis of γδ T-cell number and function in elderly people and in centenarians has demonstrated an age-dependent alteration of γδ T-lymphocytes, with a lower frequency of circulating γδ T-cells, an altered pattern of cytokine production, and an impaired in vitro expansion of these cells (Argentati et al. 2002). The decrease in the γδ T-cell number was due to an age-dependent reduction of Vδ2 T-cells, whereas the total number of Vδ1 T-cells was unaffected by age. As a result, the Vδ2/ Vδ1 ratio was inverted in old subjects and centenarians. A higher percentage of γδ T-cells producing TNF-α was found in old donors and centenarians whereas no age-related difference was observed in IFN-γ production. After in vitro expansion, a 2-fold lower expansion index of γδ T-cells, and particularly of the Vδ2 but not of the Vδ1 subset, was found in old people and centenarians in comparison with young subjects demonstrating the existence of a proliferative defect in γδ T-lymphocytes from aged subjects. In contrast, the cytotoxicity of sorted γδ T-cells was preserved in old people and centenarians. Interestingly, these cells were found more activated in the elderly than in young subjects, as determined by the increased expression of the early activation marker CD69 on γδ T-lymphocytes from old subjects, suggesting that the high level of basal activation of γδ T-cells was due to the "inflamed" environment of the elderly host (Colonna-Romano et al. 2002).

3 Immunological Targets of Breast Cancer

The immune response is a potentially useful tool in cancer prevention and treatment, and developing immunotherapies against proteins expressed on transformed cells remains a major goal of tumor immunology. Tumor-infiltrating lymphocytes obtained from metastatic effusions of breast cancer patients have been found to contain $CD8^+$ cytotoxic T-lymphocytes (CTL) that recognize autologous tumor cells in a tumor-specific, HLA Class I-restricted manner, strongly suggesting that tumor-specific antigens are present in breast cancer cells (Linehan DC 1995). Subsequent studies identified the HER-2/neu proto-oncogene and the transmembrane protein mucin (MUC1), whose expression has been correlated with poor prognosis, as important breast cancer-associated antigens. The main characteristics of these

antigens, together with those of novel breast cancer-associated antigens, such as mammaglobin-A, survivin, NY-BR-1, and nectin-4, are briefly described below.

3.1 HER-2/neu

The HER-2/neu oncogene encodes a 185-kDa receptor-like thyrosine kinase that was found to be overexpressed in several types of human adenocarcinomas, especially in breast tumours, and which was correlated with short time to relapse and poor survival of breast cancer patients (Slamon et al. 1989; Berchuck et al. 1990). It is estimated that approximately 1 in 4 breast cancers have too many copies of the HER-2 gene, resulting in the overproduction of protein receptors found on the surface of tumor cells. These special proteins bind with other circulating growth factors to cause uncontrolled tumor growth. Consequently, HER-2 positive breast cancers tend to grow fast. In healthy individuals HER2/neu receptor, which is involved in organogenesis and epithelial growth, is highly expressed during foetal development while is present at low levels in adult tissues. HER2/neu is a self antigen with poor immunogenicity due to immunological tolerance, but weak humoral (Disis et al. 1994, 1997) and cytotoxic (Fisk et al. 1995; Peoples et al. 1995) immune responses directed against HER2/neu antigen have been detected in patients with HER2/neu expressing mammary and ovarian tumours. These observations demonstrate that tolerance to this oncoprotein is not absolute and could be circumvented by using potent active vaccines enhancing to therapeutic levels the ineffective naturally occurring anti-HER2/neu immunity. In fact, in experimental models conducted in transgenic mice or in mice challenged with syngeneic tumor cells, immunization with DNA plasmids coding for p185neu, the product of the HER-2/neu oncogene, has been shown to hamper and to oppose mammary carcinoma development (Chen et al. 1998; Amici et al. 2000; Quaglino et al. 2004; Smorlesi et al. 2006).

3.2 MUC1

Human mucin (MUC) family member, MUC1, is a high molecular weight protein normally expressed in a highly glycosylated form and low levels on the apical surface of many types of normal epithelial cells (Finn et al. 1995). MUC1 is of interest and a potential target for tumor immunotherapy because it is aberrantly expressed on a wide variety of epithelial adenocarcinomas, including breast and ovarian cancer. There is an up to 100-fold increase in the amount of mucin present on cancer cells compared with normal cells; this MUC1 has an ubiquitous rather than focal cellular distribution and is present in a hypoglycosylated form, revealing peptide epitopes not easily identified in normal mucins. Such alterations are recognized by the immune system of cancer patients with MUC1$^+$ tumors. In fact, analyses of immune responses in cancer patients with various adenocarcinomas have revealed

the presence of low-titer anti-MUC1 Abs and of low-frequency MUC1-specific CTL (Kotera et al. 1994; Barnd et al. 1989). Both in experimental mouse models transgenic for human MUC-1 and in clinical studies in cancer patients, immunization against human MUC-1 increased the number of mucin-specific CTL precursors and induced some objective responses (Ko et al. 2003; Tanaka et al. 2001; Disis et al. 2002; Mukherjee et al. 2003). Higher protection was recently obtained in a transgenic mouse model expressing human MUC1; immunization of these mice with MUC1 plasmid DNA and with a plasmid encoding murine interleukin-18 (IL-18) resulted in a significant tumor protection and survival after challenge with tumor cells expressing human MUC1 (Snyder et al. 2006).

3.3 Mammaglobin-A

Mammaglobin-A has been recently identified as a novel breast-cancer associated antigen using a differential screening approach. Several properties of mammaglobin-A make it a clinically relevant breast cancer-associated marker. Unlike other genes overexpressed in breast cancer, including HER-2/neu and MUC1, mammaglobin-A is expressed at high levels in most human breast cancer cell lines and primary breast tumors (Watson et al. 1996). Furthermore, the expression of mammaglobin-A seems to be independent by breast tumor differentiation. $CD8^+$ CTL have been developed in vitro against several mammaglobin-A derived epitopes and have been found able to recognize some epitope of this tumor-associated antigen (Jaramillo et al. 2002). Using a transgenic mouse model expressing human HLA-A2 and human CD8, it has been demonstrated that vaccination with mammaglobin-A cDNA results in the development of a $CD8^+$ CTL response against mammaglobin-A^+ tumors. These $CD8^+$ CTL were able to induce the regression of established breast cancer tumors in vivo (Narayanan et al. 2004). Furthermore, $CD8^+$ T-cells generated against recombinant mammaglobin-A-pulsed dendritic cells were found to display a marked cytotoxic activity against mammaglobin-A-positive breast cancer cell lines, suggesting that mammaglobin-A can serve as a breast cancer-specific antigen and may be useful for designing new immunotherapy protocols for the treatment and prevention of breast cancer (Manna et al. 2003).

3.4 Survivin

Survivin is a member of the inhibitor of apoptosis (IAP) family, which is also involved in the regulation of cell division and is also overexpressed and associated with parameters of poor prognosis in breast cancer and in other human cancers, including carcinomas of the lung, colon, stomach, esophagus and pancreas (Sohn et al. 2006). Overexpression of survivin has been associated with resistance to chemo/endocrine therapy in breast cancer patients. Furthermore, survivin expres-

sion correlated with Grade III and lack of oestrogen receptor, and was identified as an independent predictor of shorter survival in poor prognostic breast cancer patients (Hinnis et al. 2007). The detection of circulating cancer cells expressing survivin mRNA was associated with increased recurrence of breast cancer (Yie et al. 2006). The presence of autoantibodies to survivin in the sera of patients with infiltrating ductal carcinoma of the breast (Al-Joudi and Iskandar 2006), and the activation of cytotoxic T-cells directed against survivin after in vitro culture of autologous lymphocytes with dendritic cells loaded with killed allogeneic breast cancer cells (Saito et al. 2006), demonstrated the possibility to activate both humoral and cellular immune responses against survivin, suggesting that this protein may be a suitable target for future immune-based therapeutic strategies.

3.5 NY-BR-1

NY-BR-1 is a recently identified differentiation antigen of the mammary gland which is expressed in >80% breast tumors and which elicits humoral and cellular responses in a subset of breast cancer patients (Jager et al. 2005). Furthermore, NY-BR-1 has been recently found to be more frequently expressed in grade 1 than in grade 2 or 3 carcinomas, with no difference in expression between primary tumors and metastases, and to be correlated directly with estrogen receptor expression and inversely with HER-2/neu and EGFR expression (Theurillat et al. 2007). The strong expression of NY-BR-1 in breast tumors, its cytoplasmic and membrane localization and accessibility to immune system components has suggested to pursue NY-BR-1 as a potential target for immune-based therapies in breast cancer patients (Seil et al. 2007).

3.6 Nectin-4

Nectins belong to a new family of cell adhesion molecules which are members of the immunoglobulin superfamily and are components of E-cadherin-based adherent junctions in epithelial cells (Reymond et al. 2001). Four nectins have been described which are structurally related and exibit three conserved immunoglobulin-like domains in their extracellular regions. All nectins except nectin-4 are expressed in various kinds of cells in adult tissues. Nectin-4 is mainly expressed during embryogenesis but is not detected in normal adult tissues or in serum. With regards to breast, nectin-4 is not detected in normal epithelial cells, and is highly expressed both in tumor cell lines and tumors from breast origin. Recently, it has been reported that nectin-4 is shed from the tumor cell surface and represents a sensitive serum marker for the follow-up of patients with metastatic breast carcinoma (Fabre-Lafay et al. 2007). For these reasons, nectin-4 has been suggested as a new tumor-associated antigen for breast cancer and a potential target for breast cancer immunotherapy.

4 Immune-mediated Approaches for the Prevention and Therapy of Breast Cancer in Ageing

Although newer modes of therapy for breast cancer are being applied, traditional therapy involving surgery, radiotherapy, chemotherapy, and endocrine therapy, continues to be primarily used. However, in spite of favourable prognostic factors and less aggressive biological behaviour, elderly breast cancer patients receive less aggressive treatment when compared with their younger counterparts. Patient preferences, comorbidity, functional status, life expectancy, risks and benefits of treatment, and family support are all important considerations when developing a treatment plan in older woman. Relevant therapies, such as chemotherapy, may have a role in a select group of patients with adverse prognostic factors. In fact, the pharmacokinetics behaviour of anticancer drugs may be altered with aging due to differences in body composition and decreased hepatic and renal function. For this reason, even if age is not a contraindication to cancer treatment, the administration of chemotherapy to older cancer patients involves adjustment of the dose to renal function, prophylactic use of myelopoietic growth factors, maintenance of haemoglobin levels, and proper drug selection. Adjuvant approaches based on immune system activation are promising in breast cancer treatment and, particularly in the elderly population; they might overcome the problems related to conventional treatments in the frail elderly. Immunoprevention and immunotherapy for tumor-associated antigens has become a major field of investigation for the treatment of cancer, and, particularly, of breast cancer. Cancer vaccination represents today the most intriguing possibility in activating an immune response capable of effectively hampering the progression of the preclinical stages of a tumour (Finn 2003). Cancer vaccines that can be applied in both prevention and therapy are potentially less toxic than chemotherapy or radiation and could be especially suitable for older more frail cancer patients. In recent years, experimental data have shown the effectiveness of anticancer vaccination models which can potentially elicit a potent immune response and induce immune memory against tumour antigens (Cavallo et al. 1997;Oshikawa et al. 1999; Stewart et al. 1999). Most data on the preventive potential of vaccines have been drawn from studies performed in mice transplanted with parental tumors or in transgenic mice. The use of transgenic mouse models spontaneously developing cancers is certainly preferable since murine models of cancer involving the challenge of mice with a bolus of tumour cells provide information that, while informative, may not be entirely relevant to cancer development in humans, where the tumour is initiated by the clonal expansion from a single in vivo cell. An experimental model which is widely used in studies on cancer immunoprevention is represented by murine tumours overexpressing the rat HER-2/neu proto-oncogene or its mutated transforming form. HER-2/neu transgenic mice express the activated rat neu oncogene neu-T, in which a point mutation renders the neu gene product under the control of the regulatory sequences of the MMTV promoter constitutively active. These mice develop spontaneous focal mammary adenocarcinomas beginning at 5-6 months of age, with a development kinetics and histology of these tumors that bears a striking

resemblance to what is seen in patients with breast cancer. Though the efficacy of active vaccination might be limited by the nature of HER2/neu, targeting a self tumour antigen offers the remarkable advantage in avoiding the problem of the emergence of tumour specific antigen-loss variants which are usually obstructive when targeting tumour specific nonself antigens. The genetic instability of tumour cells which can elude immune surveillance by activating mechanisms of phenotipical changes is one of the reasons why immunotherapy is ineffective (Cahill et al. 1999), but when the presence of a tumour antigen on tumour cells is the prerequisite for their tumorigenicity, as is the case with HER2/neu-expressing tumours, cells that have lost the antigen are unable to grow and cause tumours. So far the experiments performed in murine models of HER2/neu-expressing mammary carcinoma have clearly demonstrated that the efficacy of antitumour vaccination is dependent on the immunocompetence of the host (Colombo and Forni 1994; Cavallo et al. 1997). Indeed, the rejection of tumors was related to the immune effectiveness of mice and no protection against the tumour challenge was obtained in physically or chemically immunosuppressed hosts (Colombo and Forni 1994) whereas an increased antitumoral response was observed to enhance immunological effectiveness through adjuvants (Amici et al. 2000).

The remodelling of the immune system taking place during ageing suggests that vaccination models which proved efficacious in young-adult age may be not wholly efficient in old age.

In particular, at least 3 main characteristics of the immunosenescence may determine an age-related disadvantage for the potential application of cancer vaccinations in the elderly. First, the possibility of inducing an effector-cell population in response to a vaccine depends on the recognition of the vaccine antigen by naïve T-cells. It has been clearly shown that old mice give weaker primary responses than young mice, because of an age-dependent reduction of the pool of naïve T-cells and of the fact that the conversion to memory phenotype is compromised with age (Pawelec and Solana 1997; Pawelec et al. 2001; Kapasi et al. 2002). Second, in contrast to vaccines against infectious agents, in which the generation of neutralising humoral immunity is the most important feature, the major focus in cancer immunoprevention has been on the generation of Th1-cell immunity which promotes CTL responses. The ageing process appears to be accompanied by a dysregulation of Th1 and Th2 responses, with a shift to the Th2 phenotype (Shearer 1997). This dynamic change towards a Type 2-dominant state may imply that a particular vaccine strategy may not be equally efficacious in young adults and in the elderly. Third, the defect of antigen presentation by APCs to T-lymphocytes, which has been reported in aged mice, strongly suggests the existence of an age-associated multi-step defect in which the different cell populations involved in the activation of anticancer immunity are all affected (Pawelec et al. 1998; Donnini et al. 2002).

The new light shed on innate immunity and on its integration with specific immune effectors over the past few years emphasizes a further disadvantage effecting preventive approaches in the elderly. The signals that are produced by the components of the innate system required to direct the adaptive immune response may be insufficient or erroneous in aged individuals and might thus adversely influence

the specific clonal adaptive response. It is thus that the age-related alterations of γδ T-cells, NK-cells, the expression of toll like receptors on antigen presenting cells, may be relevant for their implications in the activation of inefficient specific T-cell-mediated responses.

Some direct evidence of the decreased efficiency of breast cancer vaccines in ageing have been recently described in mouse models. A study was conduced on the efficacy of interleukin-2 (IL-2)-engineered mammary tumour cells to induce an immune response capable of rejecting the tumour and of inducing specific immune memory in young and old mice (Provinciali et al. 2000). In this study it was found that mammary adenocarcinoma TS/A cells engineered to release IL-2 were rejected in both young and old mice, whereas, unlike what occurred in young mice, it was not possible to induce a specific immune memory against TS/A cells in old animals. Whereas the rejection of IL-2-transduced cells was attributed to the good infiltration of neutrophils and macrophages, the defect in memory acquisition was correlated with a reduced representation of both $CD4^+$ and $CD8^+$ lymphocytes in the tumoral infiltrate in old mice (Provinciali et al. 2000). The age-related decreased effectiveness in inducing memory against tumour cells was recently confirmed in another paper which adopted a different experimental approach. The antitumoral vaccination with DNA plasmids codifying HER-2/neu in old mice demonstrated that effectiveness in inducing protective immunity against a lethal challenge with syngeneic tumour cells overexpressing HER-2/neu was lower in old mice than it was in young animals (Provinciali et al. 2003). The reduced number of objective responses observed in old mice was associated with an age-related impairment of several immune responses. Although further evidence in other experimental models has to be provided, present knowledge suggests that the application of anticancer vaccination in ageing may not be so effective as it is in young age because of the existence of age-related defects in the activation of specific immune responses making it necessary to develop specific approaches for the immuno-prevention of cancer in advanced age.

A great bulk of experimental and clinical evidence has demonstrated that the age-related immune alteration does not represent an irreversible process and that it is possible to recover the damaged immune function through endocrinological or nutritional manipulation in old ages (Provinciali et al. 1991a, 1991b). In this context, the possibility to improve the low effectiveness of vaccination against mammary cancer in aging using “adjuvant” substances seems to represent a good approach. In fact, adjuvants are often required to augment immune responses to vaccines, particularly when the vaccine is targeting weak antigens or self-antigens, such as HER-2/neu or MUC1. One of such adjuvants might be represented by Imiquimod, an immune response modifier of the imidazo-quinoline family that has been demonstrated to exert profound anti-viral and antitumor effects (Suader 2000). The immune-modulating properties of Imiquimod have been found to be exerted through its capacity to bind to and stimulate the toll-like receptor (TLR) –7 and TLR-8, with consequent activation of the innate immune response as well as the cellular arm of acquired immunity (Stanley 2002). The potential of Imiquimod and its analogue S-27609 as adjuvants of DNA vaccination against HER-2/neu have been recently

evaluated in transgenic mice developing spontaneous mammary tumors (Smorlesi et al. 2005). The association of a DNA vaccine encoding a portion of rat HER2/neu with either Imiquimod or S-27609 was found to delay the development of spontaneous mammary tumors and to reduce their incidence, in comparison with DNA vaccination alone. Almost 80% or 40% of tumor-free mice were found at the end of measurement time in mice vaccinated and supplemented with Imiquimod or S-27609, respectively. The antitumor preventive effect was associated with increased antibody and cell-mediated immune responsiveness against HER-2/neu. In mice vaccinated and supplemented with Imiquimod, a small but significant increase of rat $p185^{neu}$-specific cytotoxicity and of IFN-γ and IL-2-producing CD8 T-cells, together with a reduction of IL-4 producing CD4 T-cells, and a switch from a IgG1 towards a IgG2a phenotype of anti-$p185^{neu}$ antibodies, suggested for a TH1 polarization of the immune response. Whether imiquimod is effective in recovering the reduced immune responsiveness observed after immunization against HER-2/neu in aged mice remains an open question even if the good adjuvant effect obtained in young age make imiquimod a good candidate for boosting aged immune functions after immunization.

Another compound that has been demonstrated potential as adjuvant of immune response in breast cancer models is the drug metformin. The treatment of HER-2/neu transgenic mice with the antidiabetic biguanide metformin inhibited mammary tumor development decreasing the incidence and the tumor size, increased their latency, and prolonged life span of these mice (Anisimov et al. 2005). The metformin effect was associated with a significant increase of cytotoxic lymphocytes producing granzyme B and perforin in their tumoral mammary glands. The exact mechanisms involved in this effect were not investigated in detail, even if an increased lymphocyte metabolism determined by the metformin-induced changes in glucose metabolism was suggested (Frauwirth and Thompson 2004).

Whereas the above described studies have demonstrated the possibility to induce a specific immune response able to prevent the development of breast cancer, the approaches the have been tried with the aim of activating an immune response able to provoke the regression of established tumours have demonstrated that rejection of an established cancer is a difficult, if not impossible, task for the immune system. The difficulty in inducing the regression of established tumors is particularly true when normally expressed “self antigens” are used as targets for human tumour immunotherapy such as, for example, HER-2/neu or MUC1 for breast cancer. This approach is based on immunologic principles which focus on circumventing tolerance, a primary mechanism of tumour immune escape. The premise for such a possibility is that the autoimmune consequences of this therapeutic approach are tolerable and not life limiting; in other words, they may effect functions that are not necessary for survival or that can be readily replaced. In murine models, the therapy of very early mammary carcinomas has been accomplished by immunizing animals against the self protein p185. Up until now these results have been obtained in murine models transplanted with HER-2/neu overexpressing tumour cells and in current studies in transgenic mouse models (Lollini a Forni 2003; Curcio et al. 2003). Immunisation of breast cancer patients with HER-2/neu peptides

generated $CD8^+$ and $CD4^+$ T-cells responsive to HER-2/neu and, as appears from the preliminary results, in some effective clinical response, without inducing autoimmunity against tissues expressing basal levels of the protein (Ko et al. 2003; Bernhard et al. 2002). In a recent study, the immunization of early breast cancer patients (ductal carcinoma in situ) with dendritic cells pulsed with HER-2/neu HLA Class I peptides increased the number of HER-2/neu-HLA-A2 tetramer-staining CD T-cells bearing CD28 antigen and decreased the inhibitory B7 ligand CTLA-4 on the same cells. The vaccinated subjects also showed accumulation of T- and B-lymphocytes in the breast and decreased HER-2/neu expression in the surgical tumor specimens, often associated with measurable decreases in residual ductal carcinoma in situ (Czerniecki et al. 2007). Studies on the immunotherapy of spontaneous carcinomas targeting the self antigen mucin-1 (MUC-1) are also in progress. Both in experimental mouse models transgenic for human MUC-1 and in clinical studies in advanced cancer patients, immunisation against human MUC-1 increased the number of mucin-specific CTL precursors and induced some objective responses (Tanaka et al. 2001; Disis et al. 2002; Ko et al. 2003; Mukherjee et al. 2003). In a recent clinical trial conducted in early stage breast cancer patients, immunization with oxidized mannan-MUC1 resulted in a significant beneficial effect, with no recurrences in patients receiving immunotherapy after more than 5.5 years after treatment. In the same study, many of immunized patients had measurable antibodies to MUC1 and MUC1-specific T-cell responses (Apostolopoulos et al. 2006).

Though there are no yet clinical data on the effectiveness of these immunotherapeutic approaches in elderly breast cancer patients, an important question is whether the immunosenescence may prove to be an advantage or disadvantage for the potential application of immunotherapeutic approaches in the elderly. Indeed, if, on the one hand, the remodelling of immune functions determines an impairment of the processes involved in immune-mediated anticancer defenses and limits the use of immunotherapy in ageing, on the other hand, the generalised phenomenon of senescence may contrast some mechanisms that favour the growth of tumours and consequently might lend support to immunological anticancer strategies. As mentioned above, the slower growth rate and the reduced aggressiveness of cancer in the old has been related to the low immune response activated in aged people against weak or nonimmunogenic tumours, like spontaneous tumours in humans; this low immune activation, even if it is unable to reject the tumour, determines a reduced production of nonspecific growth factors by immune cells which, in turn, may determine less fertile "soil" for tumour cells (Ershler and Longo 1997). Furthermore, the weaker immune response induced in old age might reduce the risk of metastatic cancer-cell clones selection caused by a powerful but incomplete immune response (Seymour et al 1999). Finally, the fact that certain poor immunogenic tumours do not grow well in old hosts but grow aggressively in young hosts has been related to age-associated deficits in tumour vascularisation and, in particular, to a lack of angiogenic factors or the presence of host inhibitors (Kreisle et al. 1990; Pili et al. 1994). A further age-related disadvantage that may contrast the activation of effective anticancer immune responses is represented by the fact that some of the mechanisms used by cancer cells to escape immune clearance might

be more effective in ageing. One of these is related to the Fas ligand (FasL)/Fas receptor (FasR) interaction. Various studies have demonstrated significant increases in the FasR expression with age, either as percentages of T-cells or as an intensity of mean fluorescence (Fagnoni et al. 2000). An increased FasR expression on aged leukocytes might facilitate the immune escape of tumours expressing FasL in elderly patients by promoting the apoptosis of tumour infiltrating leukocytes. Another mechanism which enables tumours to evade immune rejection is the release by tumour cells of immunosuppressive cytokines. Many tumours produce TGF-β, or IL-10, or other cytokines which tend to suppress inflammatory T-cell responses and cell-mediated immunity, which are needed to control tumour growth and to destruct tumour cells. In old subjects, these suppressive cytokines released by tumour cells may synergize with immunosuppressive cytokines (TGF-β, IL-10, and others) which are already overproduced by leukocytes up to elevated concentrations able to impair antitumour immune responses. Furthermore, IL-6, another cytokine overproduced in the elderly, has been reported to increase the expression of the TGF-β receptor, thus facilitating this mechanism of tumour immune escape (Zhou et al. 1993). Prostaglandins are other factors that have been involved in cancer-induced immune suppression. Tumour cells produce prostaglandins which can inhibit various immune functions. The immune suppression induced by tumour cell-derived prostaglandins may have particular implications in ageing, since lymphocytes from elderly subjects are now known to be sensitive to inhibition by prostaglandins in comparison with lymphocytes from younger individuals (Goodwin and Messner 1979).

Another piece of evidence that may influence the success of immunotherapy against self antigens in the elderly is the suspected reduced representation of cells with potential suppressive activity. In particular, it has recently reported that the number of $CD4^+CD25^+$ T-regulatory cells, a cell population capable of down-regulating immune responses to self-antigens, progressively decreases with increasing age of mice (Murakami et al. 2002). The decrease of these cells in the elderly may favour the induction of reactive immunity against self antigens rather than the activation of tolerogenic mechanisms. It seems then, that various factors may be involved in reduced tumour growth and metastatisation in the elderly particularly in the case of spontaneous tumours which express "self" antigens and are weakly immunogenic. This age-related advantage might be further exploited in the development of immunotherapeutic approaches for the elderly. A reduced T-regulatory cell number, for example, might favour the application of immunotherapeutic procedures capable of enhancing the CTL response specific for cancer associated "self" antigens.

5 Anticancer Agents as Inducers of Apoptosis and Senescence Like Growth Arrest in Breast Cancer Models

Apart from or in addition to the activation of immune-mediated anticancer effects, several agents have demonstrated effective direct antitumor activity in breast cancer models. The mechanisms involved in this anticancer action have been mainly

related to the induction of apoptosis and/or of senescence like growth arrest. For many years, the anticancer effect exerted by chemotherapeutic substances has been related to the induction of apoptosis, i.e., an active type of cell death operating in either physiological or pathological conditions in adult life and tumor regression (Vaux 1993); more recently, it has been demonstrated that an alternative pathway to cell death leading to permanent growth arrest in cancer cells may occur and may be involved in the anticancer effect of many substances. This phenomenon, called senescent-like growth arrest, is a cellular response that resembles replicative senescence occurring in normal cells and that may be crucial for protection against cancer development (Wanh et al. 2003; Narita and Lowe 2005). A commonly used surrogate marker of senescence is the senescent associated β-galactosidase (SA-β-gal) active at pH 6.0; this activity was shown to correlate with senescence in aging cell cultures in vitro and in vivo. Like other damage responses of normal cells, such as quiescence and apoptosis, senescent-like terminal proliferation arrest involves the function of wild-type p53 (Serrano et al. 1997).

A group of agents, mostly represented by natural substances, has been studied for its effect on breast cancer in in vivo murine models. The effect of these substances, mainly exerted directly on tumor cells, may certainly be useful in breast cancer treatment, having an additive effect with the antitumor immune responses induced by immunization with tumor antigens, or with immune adjuvants, particularly in old ages, when the immunosenescence may impair the efficacy of immune-mediated therapeutic approaches.

5.1 Natural Substances

5.1.1 Resveratrol

Resveratrol (*trans*-3, 4′, 5-trihydroxystilbene) is a naturally occurring polyphenolic antioxidant compound present in grapes, mulberries, peanuts, and red wine. Resveratrol has been identified as an excellent candidate cancer chemopreventive, based on its safety and efficacy in experimental models of carcinogenesis. It has been found to inhibit diverse cellular events associated with tumor initiation, promotion and progression (Jang et al. 1997). The effect of Resveratrol on the development of mammary tumors appearing spontaneously in HER-2/neu transgenic mice has been recently investigated (Provinciali et al. 2005). The mechanisms involved in Resveratrol antitumor effect were evaluated studying the immune effectiveness, the tumor apoptosis, and the expression of mRNA and protein for HER-2/neu in tumoral mammary glands from Resveratrol-treated mice and in tumor cell lines. In vivo Resveratrol supplementation delayed the development of spontaneous mammary tumors, reduced the mean number and the size of mammary tumors, and diminished the number of lung metastases in HER2/neu transgenic mice. These effects were associated with a down-regulation of HER2/neu gene expression and an increased apoptosis both in tumoral mammary glands and in murine and human

breast cancer cell lines. The induction of apoptosis caused by Resveratrol was probably consequent to its effect on HER-2/neu expression, since it has been demonstrated that apoptosis in HER-2/neu over expressing cells may be induced both by down regulating HER-2/neu expression or by inhibiting the expression or function of the p185 HER-2 protein (Roh et al. 2000). The Resveratrol supplementation did not affect immune efficiency, as neither the basal nor the IL-2-induced NK activities, nor the lymphocyte number and proliferation were modified in Resveratrol supplemented in comparison with control mice. The Resveratrol effect was then directly exerted towards cancer cells without the modulation of the immune system thus implying that the Resveratrol anticancer action may be additive to that exerted by immune-mediated therapeutic intervention.

5.1.2 Silybin

Silybin, a main component of the milk thistle of Silybum marianum, has been reported to possess anticancer activity (Kren and Walterova 2005). The effects of IdB 1016, a complex of silybin with phosphatidylcoline, were recently investigated on the development of mammary tumors appearing spontaneously in HER-2/neu transgenic mice (Provinciali et al. 2007). The mechanisms involved in IdB 1016 antitumor effect were evaluated studying the apoptosis, the senescent-like growth arrest, the intratumoral leukocyte infiltrate, and the expression of HER-2/neu and p53 in tumoral mammary glands from transgenic mice and in the human breast SKBR3 tumor cells. IdB 1016 administration delayed the development of spontaneous mammary tumors, reduced the number and the size of mammary tumor masses, and diminished the lung metastatization in HER2/neu transgenic mice. In tumoral mammary glands from IdB 1016-treated mice, a down-regulation of HER2/neu gene expression was associated with an increased senescent-like growth arrest of tumor cells, and an increased infiltrate of neutrophils, CD4 and CD8 T-cells. Both senescent-like growth arrest and apoptosis were significantly increased and were associated to a reduced $p185^{HER2/neu}$ protein and an increased p53 mRNA in SKBR3 in vitro treated with IdB 1016 in comparison with control cells. Differently with what observed using resveratrol, silybin may retain a double anticancer effect exerted at the level of both tumor cells and cellular immunity.

5.2 Hormonal Treatment

5.2.1 Melatonin

The pineal gland hormone melatonin has been shown to have an important function in the development of breast cancer. Epidemiological observations have demonstrated an increased risk of breast cancer in night shift workers, flight attendants, radio and telegraph operators, in whom an altered melatonin production is present,

and, conversely, a decreased risk in blind women. An inhibition of the pineal function with pinealectomy or with the exposure to the constant light regimen has been shown to stimulate mammary carcinogenesis, whereas the light deprivation inhibited the same (Anisimov 2003). Treatment with melatonin as well as the pineal peptide preparation Epithalamin or the synthetic tetrapeptide Epitalon was found to inhibit mammary carcinogenesis in pinealectomized rats, in animals kept at the standard light/dark regimen or at the constant illumination regimen. In HER-2/neu transgenic mice, the exposure to constant light illumination was found to promote mammary carcinogenesis whereas the administration of melatonin decreased the incidence and the size of mammary carcinomas and the incidence of lung metastasis (Baturin et al. 2001; Anisimov et al. 2002). The effects were related to the decrease of the expression of HER-2/neu mRNA determined by melatonin treatment in mammary tumors from HER-2 /neu mice. Similar effects were obtained in mice supplemented with the pineal tetrapeptide Epitalon. Besides to its direct effects on tumor cells, the anticancer action of melatonin is certainly in part linked to the modulation of immune effectiveness exerted by the pineal hormone, as previously demonstrated in various experimental models (Carillo-Vico et al. 2005).

5.2.2 Tamoxifen

The use of endocrine therapyis well established as a primary treatment for locally advanced breast cancer. Tamoxifen is an oral selective estrogen receptor modulator which is used for the treatment of early and advanced estrogen receptor positive breast cancer in pre and postmenopausal women. Tamoxifen competes with estrogen in the body for estrogen receptors in breast tissue so that transcription of estrogen-responsive genes is inhibited. Tamoxifen has traditionally been the hormone therapy of choice for patients with estrogen receptor-positive breast tumors unable to undergo surgery. However, nearly 40% of estrogen-dependent breast tumors do not respond to tamoxifen treatment and the positive response is usually of short duration because of the development of tamoxifen-resistance (Macaskill et al. 2006).

5.2.3 Aromatase Inhibitors

Aromatase inhibitors are a class of drugs used in the treatment of breast cancer that block the action of the enzyme aromatase, which converts androgens into estrogens. These drugs are generally used in postmenopausal women in whom most of the body's estrogen is produced in the adrenal gland from the conversion of androgens. Newer third-generation aromatase inhibitors, in particular letrozole, have been shown to be superior to tamoxifen in this setting with greater downstaging of tumor and disease control. The aromatase inhibitors are now the treatment of choice in elderly patients with estrogen receptor-positive breast cancer who are being considered for neoadjuvant therapy (Macaskill 2006).

6 Conclusions

The incidence of breast cancer increases with increasing age. The high rate of comorbidity in elderly patients increases the risk for complications and mortality following conventional therapies such as surgery and other adjuvant treatments, like chemotherapy and radiotherapy. Immunosenescence is a well-defined phenomenon concerning primarily the adaptive immune responses, even though some alterations at the level of most of the components of the innate pathway have been demonstrated. These age-related immune alterations play an important role both on the incidence and aggressiveness of breast cancer and on the possibility to apply preventive or therapeutic immune-based approaches in elderly patients.

Over the last few years the use of immunological measures to prevent cancer in experimental mouse models has demonstrated the possibility of preimmunising mice through new vaccines against even a poor or apparently nonimmunogenic tumour. Preventive antitumour vaccination is currently considered in humans for the prevention of the reappearance of the cancer after a primary tumour resection. On the basis of the data obtained in experimental mouse models, the strategies of immunoprevention which were effective in young-adult age do not seem to be applicable in old individuals. Attempts at finding adjuvants to improve the low effectiveness of immunisation in the elderly are needed and studies targeting this are currently being performed. It is noteworthy that the efforts aimed at designing specific protocols for the prevention and cure of cancer in the elderly should take into account either the advantages or the disadvantages offered by the senescent immune system. At the same time, anticancer substances having a primary direct action on cancer cells should be studied for their application as enhancers of immune-based strategies, particularly in old ages where the immunosenescence may impair the immune-mediated therapeutic approaches.

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Aging, Cancer and Apoptosis in Animal Models and Clinical Settings

Masanobu Kitagawa and Katsuiku Hirokawa

Contents

Keywords: Aging • Carcinogenesis • Apoptosis • Epigenetic change • DNA damage

1 Introduction

The incidence of cancer generally increases with aging of hosts in both animals and humans [39, 49], and thus advanced age is, so to say, a most powerful and potent carcinogen. In humans, the overall incidence of cancer rises exponentially in the 6th, 7th and 8th decades of life [45]. Although it is not clear what underlies this close link

M. Kitagawa (✉) · K. Hirokawa
Department of Comprehensive Pathology
Aging and Developmental Sciences
Graduate School, Tokyo Medical and Dental University
1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
Tel.: +81-3-5803-5173
Fax: +81-3-5803-0123
E-mail: masa.pth2@tmd.ac.jp

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between cancer and advanced age, it is believed that the cancer-prone phenotype of aged people is due to the cumulative mutational load over a person's lifetime. In other words, the high frequency of cancer in older individuals simply reflects a more prolonged exposure to various carcinogenic events [12]. Analysis of the frequency of human cancer as a function of age shows that between 4 and 7 mutations in key genes are usually necessary to produce cancers. However, it is still under debate whether normal mutation rates followed by the selective advantage of mutated clones are enough to produce the numerous mutations found in human cancers [106]. But, by whatever means, cancers might be caused by genetic/epigenetic alterations. Even under physiological conditions, the stem cells of our body may contain multiple somatic mutations, some of which target cancer-relevant genes [113]. For example, 1% of neonatal blood samples contain significant numbers of myeloid clones harboring oncogenic fusion of chromosomes [90] and 1/3 of adults possess detectable *IgH-BCL2* translocations associated with follicular lymphoma [85]. Thus, our body's cells experience multiple routes for oncogenesis every day. However, not so many people are affected by cancer. This may be partly because we have adequate systems that suppress carcinogenesis and constrain the growth and survival of potential cancer cells. In humans, tumor suppressor systems include the p16-Rb, ARF-p53 and telomere systems. Some of these systems protect the genome from damage or mutation. Others eliminate or arrest the proliferation of potential cancer cells by processes called apoptosis or cellular senescence. The Rb system induces cell cycle arrest and p53 and telomere dysfunction, which in turn induce apoptosis in abnormal cells. Furthermore, the immune system influences various aspects of tumor growth and metastasis [48], although there is no conclusive evidence of an immune surveillance system for carcinogenesis in humans [38].

On the other hand, aging is hastened by two major phenomena, the acceleration of cell loss and retardation of tissue repair [54, 58]. These events lead to multiorgan system functional compromise of the host, which is clinically manifested as frailty, accelerated aging, and death. Accumulating evidence suggests that dysregulation of apoptosis is associated with aging [127]. It is not clear whether such age-associated dysregulation is genetically programmed or results from nonadaptive homeostatic failure [64]. Oxidative stress and DNA damage, both important factors in aging, induce apoptosis.

The term "senescence", originally defined as a series of cellular changes associated with aging, now more commonly refers to a signal transduction program leading to irreversible arrest of cell growth, accompanied by a distinct set of changes in the cellular phenotype. Cellular senescence is a potent anticarcinogenic program, and the process of neoplastic transformation involves a series of events that allow the cell to bypass senescence [114]. Cellular senescence is controlled by the p53 and Rb tumor suppressor proteins and constitutes a potent anticancer mechanism. Nonetheless, senescent cells acquire phenotypic changes that may contribute to aging and certain age-related diseases. Thus, the cellular senescence response may be antagonistically pleiotropic, promoting early-life survival by curtailing the development of cancer but eventually limiting longevity as dysfunctional senescent cells accumulate [31, 32]. The main self-regulatory system of cell senescence, apoptosis, provides

a protective mechanism by selectively eliminating senescent, preneoplastic or superfluous cells from the body. If the regulation of this process is managed normally, apoptotic processes come at the cost of a decline in the number and proliferative reserve of stem cells, thereby suppressing tissue repair and promoting aging. In addition, if the apoptosis-inducing mechanism is dysregulated, carcinogenesis is not suppressed, resulting in the proliferation of cancer cells. Therefore, senescence-associated changes of signaling systems in cells have a significant influence on carcinogenesis. However, cellular senescence and aging of the host should be analyzed in association with cancer. The issues concerning cellular senescence are beyond the scope of this chapter, and we will restrict our discussion to the effects of aging of the host animals/humans on carcinogenesis.

Based on the understanding of the above mentioned relationship between aging and carcinogenesis, we can summarize the characteristics of changes due to aging associated with carcinogenesis as follows: 1) Increased frequency of genetic/chromosomal abnormalities [106], 2) Increased frequency of epigenetic gene silencing through DNA methylation [134], 3) Telomere dysfunction [40], 4) Decreased ribosomal RNA expression resulting in altered stromal function, which creates an environment conducive to stem cell growth [79], and 5) The deterioration of immunity by immunosenescence [29, 66, 68, 100]. We will detail animal model and human data that support this summary, and discuss the implications of the link between cancer and aging.

2 Aging and Cancer in Animal Models

2.1 Viral Carcinogenesis

There are several mouse models in which age-associated alterations in immune responsiveness are correlated with a decreased ability to cope with infection [100]. Among numerous natural pathogenic organisms of laboratory animals, viruses especially cause tumors in certain situations [20]. Using a murine experimental system of Friend leukemia virus (FLV) infection, we demonstrated an age-dependent increase in susceptibility to FLV-induced leukemogenesis [131]. This virus induces erythroleukemia in susceptible strains of mice, and the resulting tumors are highly immunogenic [35, 80, 81]. When the immunological functions of hosts was compromised by irradiation, the susceptibility to FLV-induced leukemia increased in a radiation dose-dependent manner, showing an inverse correlation with the deterioration of immune function.

First, we generated bone marrow chimeras between young and old C3H/He mice. Bone-marrow cells from young mice were transplanted to lethally-irradiated young (young → young) or old hosts (young → old), and bone-marrow cells from the old mice were transplanted to the young (old → young) or old hosts (old → old). After infection with the virus, the susceptibility of these 4 types of chimeras to FLV was compared. In young → young mice, leukemia did not develop 1 week after

FLV-inoculation, while young → old chimeras exhibited a significant increase in the nucleated cell count (NCC) in the peripheral blood. Similarly, old → young mice did not develop leukemia in contrast to the increase of NCC in old → old mice. Thus, young → old mice were more susceptible to FLV-induced leukemogenesis than young → young mice, and old → old mice were more susceptible than old → young mice. Differences in the susceptibility were not significant between young → young and old → young as well as old → old and young → old mice. The $CD4^+$ as well as $CD8^+$ T-cell populations of the spleen were reduced in chimeras with old hosts (young → old and old → old chimeras). These findings suggest that aged mice were more susceptible to FLV induced leukemogenesis, and that the major influence was due to the difference in host age and not the difference in donor age. Age-related changes in susceptibility to FLV have also been determined for other strains of mice, such as C57BL/6 and SJL [36] and the results were similar to our data. Thus, in highly immunogenic virus-induced tumors, such as FLV-induced leukemia, the deterioration of immunity due to host aging [67, 68, 99, 122] is a major factor controlling the age-dependent increase in susceptibility to viral carcinogenesis. The susceptibility-inducing mechanisms that underlie the immunosuppressive state of aged hosts probably include morphological/functional atrophy of the thymus [66, 67], age-related changes in the signaling pathways of T-cells [68, 99, 100, 122], age-related changes in cytokine producing ability of T-cells [66, 67], and age-related changes in the distribution and subpopulations of immune cells [66, 67]. Many T-cell-associated immune functions as well as antibody-producing functions of the hosts are known to control the susceptibility to FLV-induced leukemogenesis [35, 80]. For other retroviruses, genetic functions of host genes play a role in controlling retroviral diseases, including tumors, in aging in wild mice [53].

Although it is very difficult to experimentally identify the distinct immunosurveillance mechanisms in the in vivo models [118], we have a good understanding of phenomena that indicate that immune deterioration in aged hosts permits the proliferation of tumor cells in cases of human viral carcinogenesis, such as EB virus associated lymphoma [3, 91] and HTLV-1-induced leukemia/lymphoma [24, 116]. Thus, attempts to transfer immune activated cells/factors to aged or immunosuppresssed hosts might realize the control of carcinogenesis as well as tumor progression of immunogenic tumors [101].

2.2 Radiation-induced Carcinogenesis

Host aging effects have also been implicated in radiation-induced carcinogenesis. We analyzed this using a mouse model of radiation-induced thymic lymphomagenesis [123]. To separately determine the influence of aging of target cells and host environment, thymic tissues from newborn mice were transplanted to the subcapsular region of the kidney of young or old mice. Then, fractionated whole-body irradiation was performed to induce lymphoma as described earlier [77]. In young hosts, thymic lymphoma was induced in the grafted thymus as well as in the self-thymus.

However, in the old host, both the grafted thymus and self-thymus were refractory to lymphomagenesis. Thus, even in the grafted thymus, where target cells were derived from young mice, lymphoma did not develop in the old hosts. After fractionated irradiation, the young thymus exhibited a higher frequency of proliferative cells but a similar frequency of apoptotic cells compared to the old thymus. Even in the grafted newborn thymus, there were significantly fewer proliferative cells in the old host than the young host. We believe that the difference in the frequency of proliferative thymic cells is the cause of the difference in lymphomagenesis in young and old host environments.

In addition, in the rat system, the sensitivity to radiation-induced carcinogenesis is reduced with aging of the mammary glands, ovary and thyroid gland [8], which is consistent with our data. Taken together, these facts indicate that age-related factors of the target organ microenvironment, bone-marrow and whole host environment are responsible for the down-regulation of radiation-induced carcinogenesis in aged hosts, although further analysis, including measurement of cytokine or growth factor levels, is necessary to clarify the mechanism responsible for this.

In contrast to the case of viral carcinogensis, decreased immunity in the aged host cannot account for the loss of control of radiation-induced tumors. The reasons may include the quite low immunogenicity of radiation-induced tumors which do not have expression of novel proteins equivalent to those in viral infection, the potential enhancement of immune function by low dose irradiation, or the strong factor-dependent character of radiation-induced tumors.

2.3 Chemical Carcinogenesis

Some alterations characteristic of normal aging increase susceptibility to chemical carcinogens [44]. These alterations, such as a decline in DNA repair capacity and a decline in cellular immune reactivity, facilitate the induction and early growth of neoplasms. Age-dependent changes that counteract cancer development include loss of proliferative stimulation and depletion of the pool of immature cells at greatest risk. On the other hand, it has been found that genetic selection for vigorous antibody responses in most cases produces mice with longer life span and lower tumor incidence. Moreover, the results of genetic segregation experiments indicate that antibody responsiveness and life span are polygenic traits regulated by a small number of the closely linked loci. In contrast, mice genetically selected for high or low mitotic responsiveness to PHA exhibit low or high tumor incidence, respectively, but no difference in life span. These results suggest that T-cell activity is involved in immune surveillance of neoplastic transformation [42]. Actually, immunodeficient mice with a targeted disruption of the recombination-activating gene-2 (RAG2) were more susceptible to methylcholanthrene-induced tumorigenesis than wild-type mice [112]. However, whether age-related immune dysfunction influences the incidence of tumors caused by chemical carcinogens remains to be determined.

Recent studies on genoprotectors revealed that the long-term administration of the pineal indole hormone melatonin was followed by an increase in the mean life span in mice and rats [11]. In mice and rats, melatonin is a potent antioxidant both in vitro and in vivo and inhibits mutagenesis by chemical mutagens. Actually, melatonin inhibits the various types of chemically induced carcinogenesis in rodents. These experiments clearly revealed a close link between antiaging and anticarcinogenesis mechanisms, and thus aging and cancer, in experimental animals.

By contrast, in the case of hormone-dependent cancers such as breast cancer, development of chemically (N-nitrosomethyl urea) induced tumors is inversely correlated with the age of the host [88]. The function of the stroma plays a crucial role in mammary gland carcinogenesis. Thus, in certain conditions, aging effects are considered as negative factors for chemical carcinogenesis.

Overall, aging involves an increase or decrease in the sensitivity of tissues and the whole organism to the action of carcinogenic chemicals or no changes at all. These differences are due to the specific characteristics of the age-associated dynamics of the activity of drug-metabolizing enzymes and the proliferative activity of target tissues controlled by various host factors [5, 6, 7, 21, 135].

2.4 Spontaneous Carcinogenesis

The last topic we will discuss regarding carcinogenesis in animal models is the effect of aging on spontaneous carcinogenesis. The incidence of spontaneous tumors and an increase in the rate of occurrence of tumors are associated with aging in experimental animals as well as in humans [65, 89, 107]. Mutant and genetically modified animal models, which are characterized by a shortened or extended life span, offer the unique possibility of evaluating the role of gene expression in mechanisms responsible for carcinogenesis. Transgenic and knockout animal models also offer the opportunity to identify and study carcinogens and cancer-preventing agents. Generally, animal models of life span extension exhibit a longer cancer latency period than normal animals, although the incidence of spontaneous tumors is similar to that in the controls. As a result, long-lived animals are relatively resistant to spontaneous carcinogenesis [124, 125, 128]. In contrast, all models of accelerated aging have a higher incidence of tumors and the latency is shorter. Thus, aging and carcinogeneis are well correlated in experimental animal models [9, 33].

As an example, we generated *XPG* knockout mice that have a defect in DNA repair and act as a model of accelerated aging [63]. The knockout mice had small bodies and their organs were smaller in general. Furthermore, we observed frequent apoptosis in systemic organs similar to the changes in old control animals. In this model, the failure of DNA repair evokes p53 activation, frequent apoptosis in systemic organs and a phenotype similar to aging. At the same time, failure of DNA repair in this model is also associated with a higher incidence of UV-induced as well as spontaneous cancers.

Telomeres are specialized nucleoprotein complexes that serve as protective caps at the ends of linear eukaryotic chromosomes. Telomere dysfunction impairs DNA repair [129], and thus enhances sensitivity to various DNA-damage-inducing signals. Therefore, telomere dysfunction in aged animals is related to aging phenotypes, the lower potential of target organ cells to proliferate and a higher level of apoptosis. Accumulation of telomere dysfunction in old animals is also related to a higher incidence of spontaneous cancers [109, 129]. The issue of telomere dysfunction will be discussed again in a later chapter concerning the human clinical setting.

2.5 Summary of Animal Models

Susceptibility to viral carcinogenesis increases with advancing age of the host. This is probably associated with a deterioration in immunity in aged hosts, because virus-induced tumors are usually highly immunogenic. However, for other tumors there is little evidence to support a direct causal link between immunosenescence and cancer development in animals or humans [29, 48]. On the contrary, radiation-induced carcinogenesis occurs at a lower frequency in aged hosts, and is influenced by host environment factors, including cytokine and growth factor production, resulting in low proliferative activity of target tissues. Chemical carcinogenesis occurs more frequently in aged hosts because of accumulated genetic abnormalities that cause instability and a lower ability to metabolize carcinogens. However, the relationship between the incidence of chemically induced tumors and age is not uniform, and depends on the characteristics of chemicals and the mechanisms by which they induce tumors. Finally, spontaneous cancer is usually more frequent in the aged host, both for normal animals and genetically modified animals. The accumulation of genetic mutations as well as telomere dysfunction is associated with a higher incidence of spontaneous cancers in old animals. The overall influence of the aging effect on tumor induction in animal models is controversial because multiple factors contribute to the susceptibility to carcinogenesis (Table 1).

Table 1 Host aging effects on carcinogenesis: summary of animal models

Manner	Susceptibility	Age-related mechanisms
Viral carcinogenesis	Increased	Deterioration of immunity against highly antigenic tumor
Radiation-induced carcinogenesis	Decreased	Altered host environment, low proliferative activity of target tissues
Chemical carcinogenesis	Increased/Decreased	Gene instability, low metabolic activity, inadequate hormone production
Spontaneous carcinogenesis	Increased	Frequent gene mutations, telomere dysfunction
Overall	Increased/Decreased	Multifactorial

2.6 Differences in Cancer Development between Animals and Humans

Information obtained from animal models has contributed substantially to the development of treatments for human cancers. However, important interspecies differences have to be taken into account when considering the mechanisms of cancer development and extrapolating the results from animals (mainly mice) to humans [10]. The essential differences in cancer development between mice and humans include 1) tumor origin (commonly mesodermal sarcomas in mice compared with epithelial carcinomas in humans), 2) carcinogenic risk factors (many mouse carcinogens are noncarcinogenic in humans and vice versa, probably due to differences in the basal metabolic rate and metabolic pathway in the liver), 3) the number of genetic events necessary to induce malignant transformation (fewer genetic events are required in mice), 4) spontaneous regression of tumors (occurs in infants but is rare in adult humans, whereas it is common in adult mice) and so on. The intracellular wiring mechanisms of cells also differ regarding telomere biology, regulation of cellular senescence by p53 and Rb, and the RAS pathway [60,104]. Moreover, after a steady increase during adult life, the cancer incidence rate decelerates or even declines in very old age (above 70 in humans) for most sites of cancer development in both animals and humans [10]. This fact might reflect important commonalities in the basic mechanisms of age-specific predisposition to cancer among different mammalian species. It might indicate that aging, as a fundamental process, affects susceptibility to cancer similarly in humans and animals. Therefore, differences in the manner of cancer development do not diminish the importance of animal model analysis.

3 Aging and Cancer in the Clinical Setting

3.1 Types of Tumors Characteristically Observed in the Elderly

Next, we will analyze the characteristics of human cancers in the elderly in comparison with those in younger populations. Common cancers the incidence of which increases with age include prostate, colon and lung cancers, which originate from epithelial cells. From the discussion of animal models, age-dependent genetic and epigenetic events are considered likely to contribute to the increased incidence of these cancers in the elderly. Less evident is how such events spur the preferential development of such epithelial cancers in the elderly, while sarcomas and some types of lymphomas generally predominate in younger and even pediatric populations. A recent study using a telomerase-knockout mouse indicated that differences in telomere length and regulation might impact the spectrum of tumors during aging [18]. Conversely, constitutive telomerase expression promotes mammary carcinomas in aging mice [19]. In contrast to humans, the laboratory mouse possesses long

telomeres, and thus the overexpression of telomerase might promote shortening of telomeres. From the viewpoint of the telomere length, this means the introduction of a human-like condition in mice. Compared to tumors that arise in mice with intact telomeres, tumors in mice with telomere dysfunction possess higher levels of genomic instability and show numerous amplifications and deletions in regions syntenic to human cancer hotspots [96]. Although it is unknown whether these data from telomerase-modified mice are relevant to mechanisms of epithelial carcinogenesis in the elderly, telomere dysfunction can be used to interpret the specificity of cancer distribution in the elderly. From the point of view of immunosenescence, differences in the immunogenicity of tumor types would explain the selectively higher incidence of epithelial tumors in the elderly. In other words, the incidence of highly immunogenic tumors would be more directly influenced by immunosenescence than that of weakly immunogenic tumors. Although even spontaneous tumors such as melanoma are immunogenic [101], overall immunogenic properties do not correrate with the specific tumor types commonly observed in the elderly [78, 112].

We next discuss hematological malignancies and gastrointestinal cancers as well as the specificity of cancers in the elderly.

3.2 Effects of Aging on Hematopoiesis with Reference to Myelodysplastic Syndromes

Anemia is an issue of concern for the management of older patients with various diseases. The prevalence of anemia gradually increases after age 60 [4]. The prevalence and incidence also start increasing by age 65, with steeper increases after age 80. The hematopoietic reserve may become compromised due to a number of factors, including a reduced concentration of hematopoietic stem cells, reduced sensitivity of stem cells and hematopoietic progenitors to growth factors, increased circulation of substances that inhibit hematopoiesis in the circulation and in the hematopoietic microenvironment, and compromised ability of the microenvironment to support and nurture these elements [22]. Among these, a decline in the number of pluripotent hematopoietic stem cells and a decline in the production of hematopoietic growth factors are supported by data that are inconclusive at best [23]. The best evidence suggests that aging is associated with increased levels of circulating cytokines such as IL-6, which may compromise the response of stem cells and hematopoietic progenitors to growth factors [61], although currently available experimental evidence is not conclusive regarding this [103]. To assess the overall effects of aging-associated factors, we examined the change in cellularity of the bone marrow with age [95]. Bone-marrow samples from the iliac/sternal bone show stable levels of cellularity in the young, middle-aged and even in old age at < 80 years. However, the bone-marrow shows significantly hypoplastic features in people over age 80. These features are consistent with the findings that the prevalence and incidence of anemia starts increasing by age 65 and is especially high after

age 80 [4]. The question then, is what causes the hypocellularity of bone-marrow in the elderly over 80? First, we investigated the proliferative activity of the bone-marrow cells. The change with age was not striking for those under 80, although the activity was slightly reduced in those over 80. In contrast, a remarkable increase in the apoptotic cell frequency was observed in the bone marrow of the elderly. Therefore, apoptotic cell loss rather than the reduced proliferation of bone-marrow cells leads to the hypocellularity of bone-marrow in the elderly, and thus the high frequency of apoptosis is an important characteristic of aged bone-marrow.

Myelodysplastic syndromes (MDS) are hematological malignancies which mainly affect the elderly. The bone-marrow cells of MDS patients exhibit frequent apoptosis [83] which causes ineffective hematopoiesis resulting in cytopenias of the peripheral blood in spite of normo- to hyperplastic bone-marrow. Therefore, frequent apoptosis is a hallmark of the bone-marrow of the elderly under normal and neoplastic conditions. Many factors are associated with frequent apoptosis in the MDS bone marrow. We have shown that the TNF/TNFR, Fas/FasL, and NO systems are all involved [82, 83, 84, 110]. However, when the apoptotic machinery is suppressed [132], the proliferation of more malignant cells occurs in the bone-marrow of MDS, thereby causing the evolution of overt leukemia. This correlation between apoptosis and malignant transformation in MDS bone-marrow is well documented together with correlations with aging phenomena (apoptosis of multiple organs) and carcinogenesis (suppression of apoptosis) in the elderly.

3.3 Gastrointestinal Cancer in the Elderly

Concerning carcinomas of the gastrointestinal tract in the elderly, clinicopathological studies have pointed out several characteristics in these patients. Gastric cancers in aged patients tend to be distributed over the lower one-third of the stomach, show histological features of well-differentiated adenocarcinoma, and exhibit a higher incidence of multiple cancers in the stomach [16, 50, 69]. Old patients show a lower incidence of peritoneal involvement and lymph node metastasis of gastric cancer as compared with younger patients [51]. Duodenal cancers in the elderly frequently occur in the first portion of the duodenum in contrast to the majority of primary duodenal cancers, which are found in the second portion in younger patients [13]. Colorectal cancers in the elderly occur more frequently in the proximal colon (proximal to the splenic flexure) than in younger people and the ratio of proximal colon cancer increases with advancing age. Higher proportions of poorly differentiated adenocarcinoma, mucinous carcinoma, cancer >5 cm in size, and protruding-type cancer are present in the elderly, although these kinds of tumors typically occur in the proximal colon [14]. The incidence of multiple cancers shows no age-related difference for colorectal cancer [15].

Many naturally occurring tumors in humans and experimental animals show slower growth with advancing age [76, 87, 102]. In the case of poorly antigenic tumors, the immune system might transduce stimuli responsible for the proliferation

rather than the elimination of tumor cells. Thus, age-related hypofunction of the immune system may contribute to slower tumor growth in cancers of the elderly [47, 76]. Another mechanism involves vascular factors, whereby transplanted tumors in animal models grow and spread less readily in older hosts due to a reduced capacity to vascularize the tumors [102]. We next introduce our data on the biological aspects of colon cancers in the elderly. To clarify the cell dynamics of colon cancer cells in the elderly *in vivo*, we analyzed cell proliferation and apoptosis in colon cancers of old people. Ki-67-positive proliferative cells increased with advancing age, and at the same time the TUNEL-positive apoptotic cell ratio increased with aging in normal as well as in cancer tissues [119]. Therefore, the slow growth of colon cancers in the elderly may be related to an increase in apoptotic cells rather than being associated with the proliferative activity of tumor cells, because the rate of cell proliferation is higher in cancer tissues of elderly compared to younger patients. We confirmed that molecules which may be associated with cell growth, such as c-myc, were more strongly expressed in cancer tissues from elderly than younger patients [111]. Furthermore, apoptosis-enhancing molecules such as Bak and Bax were more strongly expressed in the tumors from the elderly [111, 119]. We also examined the expression of apoptosis-inhibitory proteins in colon cancer. We expected that apoptosis-prone cancer of the elderly would show lower expression of inhibitor of apoptosis protein (IAP), but our results were contrary to this. Colon cancers from the elderly exhibited a higher expression of apoptosis-inhibitory proteins of the IAP family such as survivin and cIAP2 compared with those from younger patients [46]. Immunohistochemical staining also revealed strong expression of surviving in cancers of the elderly. The question then is, what causes the frequent apoptosis in colon cancers in the elderly? In general, apoptosis-causing signals such as cellular injury induce the up-regulation of Bak and Bax, and this reaction is suppressed by Bcl-2 [121]. These signals stimulate mitochondria to induce caspase-dependent and caspase-independent pathways associated with apoptosis. Bak/Bax expression is higher and Bcl-2 expression is lower in colon cancers in the elderly than in cancers in the young [119]. Therefore, stimulation of the mitochondria to induce apoptosis is stronger in cancers in the elderly. However, IAP proteins are also up-regulated in these cancers. Thus, the caspase-dependent apoptotic pathway is suppressed by IAPs, and the caspase-independent apoptotic pathway [1] is important for inducing apoptosis in the elderly. Further studies should clarify the contribution of recently identified molecules such as AIF or Endo G to this process [37, 41].

From these findings, we hypothesized that old patients with colon cancer have a better prognosis than younger adult patients because the growth of tumors should be slower, but this also was not the case. We followed the prognosis of patients with colon cancer who underwent surgery for the resection of tumors. When they were divided into 2 groups comprising those over 80 and those under 80, the older group exhibited a significantly worse prognosis than the younger group. To eliminate other factors associated with aging, we selected only Dukes B group patients and patients who apparently died of cancer, but the results were the same. Older patients exhibited worse prognosis than did younger patients [92]. Thus, the behavior of cancers in the elderly is complicated. One explanation for the poor survival of colon cancer patients

among the elderly is the late manifestation of clinical symptoms, which might be caused by the slower growth of tumors or other factors such as lower sensitivity of the host to abnormal functions. Colon cancers in the elderly tended to be in an advanced stage at the time of initial diagnosis, showing deeper invasion and a higher frequency of metastases. This partly explains the poor prognosis of colon cancer patients among the elderly. Patients with slow growing tumors have a better prognosis when we see the whole course of the disease. However, because we have the tools necessary to treat cancers by surgical resection, slow growing tumors do not always mean a good prognosis. Finally, we have to consider the influence of immunosenescence. Lower ability to immunologically eliminate cancer cells in the elderly might permit the progression of the primary tumor as well as metastatic tumors. This might also influence the poor prognosis of colon cancers in the elderly.

3.4 Genetic Disorders and Cancer

There are numerous genetic disorders marked by chromosome instability that are associated with various cancers. Chromosomal instabilities and neoplastic outcomes are related to abnormalities of DNA metabolism, DNA repair, cell cycle activity and the control of apoptosis. Among these diseases are ataxia telangiectasia and Nijimegen breakage syndrome, which are associated with an increased incidence of lymphomas. Bloom syndrome, Werner syndrome, and Rothmund-Thompson syndrome, each characterized by a DNA helicase defect, are associated with early incidence of different types of cancers. Other diseases that combine phenotypes associated with chromosomal instabilities and neoplastic development are Fanconi anemia and breast cancers associated with mutant BRCA1 and BRCA2 genes [43]. In these disorders involving DNA recombination, some DNA helicase defects are associated with aging, although the exact pathways that link the mechanisms responsible for the genetic defects to the eventual development of various cancers as well as early aging remain to be elucidated.

3.5 Werner Syndrome: Accelerated Senescence and Cancer

Werner syndrome (WS) is an autosomal, recessively inherited segmental progeroid syndrome in which patients appear much older than their chronological age and exhibit many of the clinical signs and symptoms of normal aging at an early stage in life [55, 57]. They develop many age-associated diseases early in life, including atherosclerosis, osteoporosis and cataracts, and display a high incidence of cancer. This mimicry of normal aging has made this syndrome a focus of recent molecular studies on the pathophysiology of aging.

WS is caused by a mutation in *WRN*, which is a member of the *RECQ* family of DNA helicases [133]. WRN helicase associates with proteins involved in DNA

transactions, including those that resolve alternative DNA structures or repair DNA damage. The biochemical activities of WRN and the functions of WRN-associated proteins suggest that *in vivo* WRN resolves topological or structural DNA aberrations that occur during DNA metabolic processes such as recombination, replication and repair or that are the result of DNA damage [115]. However, some features of WS are also present in patients with other mutations such as laminopathies caused by mutant *LMNA* encoding nuclear lamin A/C [34]. Thus, WS may be a molecularly heterogeneous disease.

About 80% of WS cases worldwide are Japanese, which is probably due to inbreeding and to the background of high *WRN* mutation rates in Japan [70, 71, 108]. Surveys of Japanese patients [56] revealed that WS is associated with a high risk of a spectrum of rare neoplasms rather than the accelerated occurrence of ordinary cancers. They include 1) nonepithelial malignant or premalignant tumors/conditions; osteosarcomas, soft tissue sarcomas, malignant melanomas, myeloid leukemia and myelodysplastic syndromes, 2) an epithelial neoplasm; thyroid carcinoma, and 3) meningiomas, although the majority of cases are benign. Common carcinomas of the aged, for example, lung, colon and prostate cancers, are rare. Thyroid carcinoma, the most frequent epithelial cancer in WS, accounts for 14% of neoplasms in Japanese with WS. The ratio of epithelial to nonepithelial cancer is 1:1 compared with 10:1 in the general adult population. Germline mutations may be related to cancer with atypical features, in that they differ in their distribution according to age, gender, anatomic site and/or histologic type [98].

WS is also marked by increased genome instability manifested as chromosomal alterations. Characterization and analysis of the WRN protein suggests that it participates in several important DNA metabolic pathways, and that its primary function may be in DNA repair [97]. Thus, the WRN protein represents an important link between defective DNA repair and processes related to aging and cancer. The relationship between the failure of DNA repair and aging/carcinogenesis can also be observed in animal models, as we showed for *XPG* knockout mice [63].

3.6 Dyskeratosis Congenita: Telomere Dysfunction Linking Aging and Cancer

Telomeres consist of small tandem nucleotide repeats that are located at the ends of chromosomes and operate to protect the chromosomes from end-to-end fusions. Due to the "end-replication insufficiency" of DNA polymerase, telomeres shorten during each round of cell division. Telomere erosion below a certain length can then trigger apoptosis of the cell. Therefore, telomere shortening limits the proliferative capacity of cells and restrains the regenerative capacity of organ systems during aging, leading to age-related phenotypes such as reduced wound healing and a weakened immune system. Telomere shortening apparently has a dual role in tumor development and progression. On the one hand, it induces chromosomal instability and the initiation of cancer; on the other hand, tumor progression requires stabilization of

telomeres [109]. The predominant mechanism of telomere stabilization in tumor cells is the activation of the telomere-synthesizing enzyme telomerase.

Some of the most persuasive data on this point comes from patients suffering from dyskeratosis congenita [117]. These patients have lost telomerase function, resulting in a defect of the preservation of telomere length. Analysis of cells from dyskeratosis congenita patients reveals telomere shortening and dysfunction compared with that in the cells of age-matched controls. Patients suffering from this disease manifest several distinct abnormalities, including abnormal skin pigmentation, nail dystrophy, mucosal leukoplakia, bone-marrow failure and cancer disposition.

Thus, telomere shortening results in phenotypes of aging and also causes genomic instability that is believed to be a driving force in the cell transformation process. The cellular response to telomere dysfunction—senescence and apoptosis—might contribute to aging phenotypes [105]. However, it is only in cells that have lost the checkpoint functions (tumor suppressor functions) that involve apoptosis and senescence that telomere dysfunction can lead to the genomic instability that fuels cancer.

3.7 Epigenetic Changes in Human Cancers and Aging

Because the alterations of gene function in cancer cannot be explained by mutations alone [106], a nonstructural mechanism may exist as well. Some cancers show hypermethylation of gene promoters resulting in the loss of gene function. DNA methylation patterns can be inherited when cells divide. This epigenetic process as an alternative to mutations can inhibit tumor suppressor gene function [25,74]. Thus, aberrant DNA methylation is a powerful mechanism for the abolition of gene activity and is observed in various cancers. Other examples of genes silenced by DNA methylation include imprinted genes and genes on the inactive X chromosome in female mammals [86, 130].

Although three major methyltransferases have been cloned from mammalian cells [27], the molecular mechanisms involved in transcriptional silencing through DNA methylation have not been determined. However, the following mechanisms have been suggested [25]. Dense methylation reduces the binding affinity of sequence-specific transcription factors. In addition, methyl DNA-binding proteins may exclude transcriptional machinery and interfere with RNA polymerase activity. Recently, the functional implications of the association of methylation with transcriptionally repressive chromatin were outlined. Methyl DNA-binding proteins bind to methylated DNA and recruit histone deacetylase and transcriptional corepressors. The deacetylation of histones then reduces transcription by allowing tighter nucleosomal packing [134].

There are 3 types of cancer-related genes: oncogenes, tumor suppressor genes and DNA repair genes. Several tumor suppressor genes and DNA repair genes have been reported to be methylated in various cancers [52, 75] and include *APC*, *BRCA1*, *CDH1*, $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and *WT1* as tumor suppressor genes and *hMLH1*, *GSTP1* and *MGMT* as DNA repair genes. Hypermethylation of other genes, including *COX-2*, *DAPK* and *TIMP3*, has also been reported for several

types of cancers. Some of these genes are simultaneously methylated in the same tumor and are methylated in various cancers, while others show cancer-type-specific hypermethylation [52]. Genetic changes such as point mutations, insertions, deletions and allelic losses are involved in the inactivation of tumor suppressor and DNA repair genes. However, the discovery of many hypermethylated promoters of tumor suppressor genes indicates that DNA methylation is an important alternative mechanism for gene inactivation in many cancers [134].

Aging, chronic inflammation, and viral infections are known to promote methylation of noncore regions of promoter CpG islands. The noncore methylation is considered to serve as trigger for dense methylation of promoter CpG islands, which permanentaly repress expression of their downstream gene. In the normal colon mucosa, DNA hypermethylation of the *estrogen receptor (ER)*, *CSPG2*, *IGF2*, *MYOD1* and *N33* genes was observed to have occurred in a subpopulation of cells, which increased with advancing age [2, 72]. Furthermore, age-related hypermethylation was also demonstrated in the *DBCCR1* gene in the normal urinary bladder [59] and in the *Hic1* gene in the prostate. Increased age-related DNA methylation was found in the colonic mucosa of patients with ulcerative colitis [73], suggesting that chronic inflammation is associated with high levels of methylation, probably as a result of increased cell turnover.

These genes affected by age-related methylation in normal tissue are also methylated in primary cancers that originate from the same tissue [134]. Therefore, in some tissues, age-related methylation starts in the normal mucosa as a function of age, and then may suppress gene function, possibly in association with carcinogenesis of the primary cancer. However, in many organs cancer-related methylation is restricted to cancer tissues and no methylation is observed in normal tissues. Although DNA methylation is not a general or direct factor that links aging and cancer, further studies should clarify the role of epigenetic modification of DNA in association with aging as well as carcinogenesis.

For the *hMLH1* gene, which is a member of the DNA mismatch repair gene family, methylation was only found in cancers and not in the normal tissue [120]. Patients with gastric cancers showing aberrant *hMLH1* expression and methylation are significantly older than those with cancers without these aberrant phenotypes. The prevalence of aberrant hMLH1 expression and methylation significantly increases with advancing age. Thus, *hMLH1* methylation is not only cancer-specific but also age-related in cancers. It is also likely that *hMLH1* methylation plays a role in gastric carcinogenesis in the elderly [93]. Furthermore, a recent study found that *hMLH1* hypermethylation also plays a role in the development of medullary-type poorly differentiated colorectal adenocarcinomas in the elderly [17].

3.8 Altered Expression of MicroRNA (miRNA) in Aging and Cancer

Remarkable progress in embryonic and adult stem cell research in the past several years has yielded a wealth of information regarding the mechanisms regulating self-renewal and differentiation, two processes often used to decline stem cells.

Some investigators speculate that aberrant epigenetic events as well as altered microRNA (miRNA) expression in aged stem cell populations play important roles in carcinogenesis [94]. Stem cells retain the expression of miRNAs that are important to maintain the stemness state [62]. Data from recent studies suggest that miRNA-mediated carcinogenesis results from either down-regulation of tumor suppressor and/or up-regulation of oncogenes [30]. Thus, miRNAs that are important in maintaining stem cell identity also seem to be important in cancer development. It remains to be clarified how miRNA expression patterns change as a function of aging.

4 Apoptosis: the Key Phenomenon Linking Aging and Carcinogenesis

4.1 Overview

Animals, including humans, have evolved strategies—tumor suppressor mechanisms—to suppress the development of cancer. It is clear that at least 2 strategies have evolved to suppress cancer. One is so-called "caretaker" proteins that prevent cancer by protecting the genome from acquiring potentially carcinogenic mutations. By contrast, "gatekeeper" tumor suppressors prevent cancer by acting on intact cells to eliminate or prevent the growth of potential cancer cells [31]. However, recent evidence indicates that some mammalian tumor suppressor mechanisms contribute to aging. Namely, the gatekeeper causes apoptotic and senescence responses that may limit longevity by contributing to aging and late-life pathology. In the case of apoptosis, this process could eventually deplete nonrenewable tissues of irreplaceable postmitotic cells and renewable tissues of proliferating or stem cell pools. The senescence response could likewise deplete tissues of proliferating or stem cell pools. Furthermore, senescent cells are dysfunctional and may actively disrupt normal tissues as they accumulate. Therefore, in this chapter we overviewed the mechanisms of aging and carcinogenesis with special reference to apoptosis.

Although many hypotheses have been proposed to explain the strong link between aging and cancer, as described above, the exact mechanisms responsible for the increased frequency of cancer with advancing age have not been fully defined. Recent evidence indicates that dysregulation of apoptosis may contribute to certain aging processes [127]. On the other hand, apoptosis provides a protective mechanism by selectively eliminating gene-mutated, senescent, preneoplastic or superfluous cells. If the regulation of this process is managed normally, apoptotic processes come at the cost of a decline in stem cell number and their proliferative reserve, thereby suppressing tissue repair and promoting aging. In addition, if the apoptosis-inducing mechanism is dysregulated, carcinogenesis is not suppressed, resulting in the proliferation of cancer cells. Although how apoptosis is altered during aging in vivo is still being debated, this phenomenon is a key candidate for dissecting the complicated mechanisms that link cancer and aging.

During aging, we observed 1) increased apoptosis in normal/tumor tissues, 2) the accumulation of genetic/epigenetic abnormalities and telomere dysfunction, and 3) increased spontaneous cancer (epithelial).

Regarding the mechanism that links aging and spontaneous carcinogenesis, we propose the mechanism shown in the flow chart of Fig. 1, whereby stress, DNA-damage, telomere dysfunction, etc., produce abnormal cells including preneoplastic cells, resulting in the activation of the apoptotic machinery. If the regulation of apoptosis is normal, this process leads to aging effects. However, once this system is malregulated via various factors [126], the risk of cancer increases. Although it may seem paradoxical that aging and cancer both originate from the same processes involved in maintaining the viability of the cell/host, the apoptosis-inducing mechanism is a key factor that links aging and cancer. However, we should also take into account the influence of immnosenescence on carcinogenesis in the elderly. We can detect the immunosurveillance phenomenon [112] and even spontaneous tumors such as melanoma are immunogenic and are commonly infiltrated by tumor antigen-specific T-cells [101]. Thus, especially in immunogenic tumors, immunosenescence would help to promote carcinogenesis in the elderly, as observed in animal models [131] and human viral diseases [3, 24, 91, 116].

4.2 Role of Mitochondria in Aging and Cancer

Mitochondria play roles in multiple cellular functions, including energy production, cell proliferation and apoptosis. These organelles contain their own genetic material, mitochondrial DNA (mtDNA), which is maternally inherited. Although much smaller than the nuclear genome, mtDNA is also important, and has been hypothesized to play a crucial role in aging and carcinogenesis. This is partly due to the fact that mitochondria represent the major site for the generation of cellular oxidative stress and play a key role in mediating apoptosis. Damage to mtDNA is therefore an important contributor to aging and cancer [28].

The free radical theory remains the most vigorous contender to explain the basis of aging in a wide range of species by postulating that the production of intracellular reactive oxygen species (ROS) is the major determinant of life span [28]. Intracellular ROS are primarily generated by the mitochondrial respiratory chain and are prime agents of oxidative damage. Free radical damage is generated and accumulates during normal metabolism and in stress situations. Persistently high ROS is linked to several age-related diseases. Furthermore, recent data from elegant mouse models now confirm that mutations of mtDNA do indeed play a central and pivotal role in the aging process [26].

One important development has been the recognition that mitochondria play a central role in the regulation of apoptosis. A number of apoptotic signals converge on mitochondria. Thus, mitochondria have been implicated in the carcinogenic process because of their role in apoptosis and other aspects of tumor biology, and also because of their role as generators of ROS (Fig. 1). Many types of human malignan-

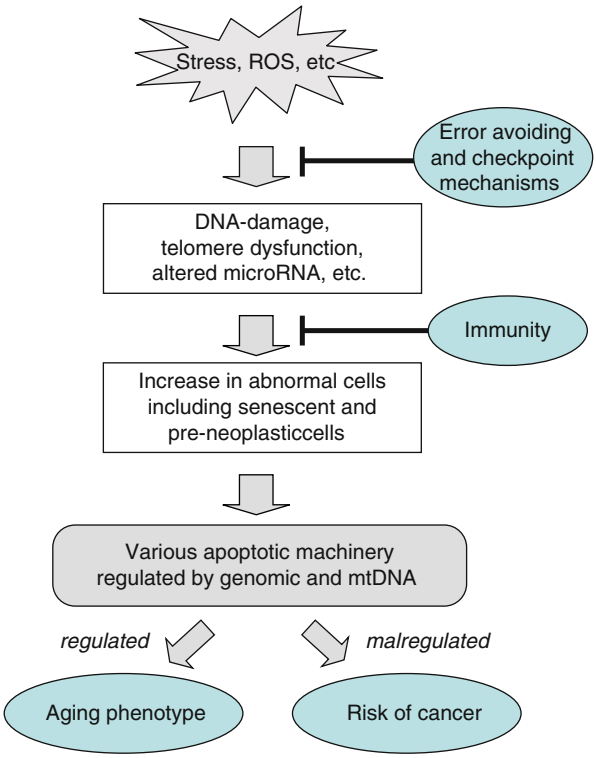

Fig. 1 Mechanisms linking aging and carcinogenesis The activation of various apoptotic machinery in the aged plays a role in the manifestation of aging effects and the development of cancer.

cies, such as colorectal, liver, breast, pancreatic, lung, prostate, bladder and skin cancer, have been shown to harbour somatic mtDNA mutations [28]. It is currently unknown whether the observed mtDNA damage has a primary and causative link to the process of cancer development or if it may simply represent a secondary bystander effect that reflects an underlying nuclear DNA instability.

In conclusion, it is likely that the interplay between nuclear and mitochondrial genes may hold the final key to understanding the role of mitochondria in aging and cancer.

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Her-2/neu Transgenic Mice for Evaluation of Immune and Antitumor Responses Against Self-Tumor Antigens in the Young and the Old

Joseph Lustgarten and Noweeda Mirza

Contents

Abstract: The impact of aging on T-cell tolerance has yet to be elucidated. More importantly cancer vaccines that will be effective both in the young and the old have yet to be developed. As a result, there is a need for relevant tumor models which include aspects of self-tolerance and development of spontaneous tumors in the aged. Such models are critical for the development and optimization of specific cancer-related immunotherapeutic strategies for the elderly. Although the majority of studies augmenting immune responses against a self-antigen like the Her-2/neu have used young Her-2/neu transgenic mouse models and put a lot of effort into aspects such as increasing the immunogenicity, very little attention has been paid to the immune competence of the aging population. Based on the Her-2/neu transgenic mice, our

J. Lustgarten (✉) · N. Mirza
Cancer Center Scottsdale
Mayo Clinic Arizona
13400 East Shea Boulevard
Scottsdale, AZ 85259, USA
E-mail: lustgarten.joseph@mayo.edu; mirza.noweeda@mayo.edu

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group has developed a mouse model (HLA-A2.1/Kb mice crossed with the FVB-Her-2/neu mice) where self-tolerance, spontaneous tumor progression and aging are present simultaneously. The immunological aspects of the A2xneu mice closely reflect those of cancer patients whose immune systems are not fully competent to reject their tumors. Models like this are critical as they may provide data that closely predict the clinical outcomes and will help to customize immunotherapeutic strategies that would be effective for the treatment of tumors in both the young and the old. In this chapter, we will focus on the Her-2/neu transgenic mouse model for the evaluation of immune and antitumor responses against a self-tumor antigen in both the young and the old.

1 Tumor Associated Antigens

Immunotherapeutic strategies designed to induce a cellular immune response have received much attention as a promising approach for the treatment of many types of cancers. The discovery of tumor associated antigens (TAA) [90, 179, 178] has been an important breakthrough in tumor immunology, because it is now possible to devise immunotherapeutic approaches to promote T- and B cell responses against such antigens and induce protective immunity against neoplastic malignancies [39, 176]. TAA can be classified into four categories based on their expression and recognition patterns of T-cells [137]. The first family is known as cancer-testes antigens (CTAs). These proteins are normally expressed only in testes but are aberrantly expressed in melanoma, bladder, colon, lung, prostate and other cancers. The NY-ESO-1 [19] and the MAGE families [180] are proteins that characterize this group. The second family is known as differentiation antigens, like the melanocyte lineage [77]. These antigens show a lineage specific expression in tumors (melanomas) and are also expressed in normal cells of the same origin. The tyrosinase [10] or gp100 antigens [195] are examples of this group. The third family of antigens are viral-based proteins. These are cancers induced by viruses like the human papillomavirus (HPV 16) that induce cervical cancer [162]. Antigens such as E6 and E7 from HPV 16 can be recognized by T-cells and used as targets for tumor protection [42]. The fourth family are "self-antigens" that are overexpressed in the tumor compared to the level of expression in normal cells [113]. The Her-2/neu [69] and p53 [64] are examples of this family. These antigens can be used to target a wide range of tumors from different origins. This chapter reviews basic information on targeting Her-2/neu for cancer immunotherapy in the young and the old.

2 Her-2/neu

Breast carcinoma is a biologically complex disease that ranges from a localized tumor to a widely varied metastatic neoplasm. Expression and amplification of oncogenes have been studied in attempts to define the molecular correlation of the prognosis,

progression and clinical behavior of breast cancer. A major genetic alteration in breast cancer is the overexpression of the Her-2/neu (also known as c-erbB-2) proto-oncogene [27, 1, 7]. This protein is expressed in 25-35 % of all breast cancers. Her-2/neu is a transmembrane glycoprotein with tyrosine kinase activity whose structure is similar to epidermal growth factor receptor (EGFR) [126]. Her-2/neu is a component of a four member family of closely related growth factor receptors including EGFR, Her-1, Her-3 and Her-4 [181]. Her-2/neu is involved in the regulation of vital functions including cell proliferation and cell differentiation, therefore, these effects can initiate a hyper-mitogenic signal with oncogenic potential [174]. The presence of Her-2/neu on tumors is associated with metastatic disease, poor prognosis and overall survival [156]. One of the consequences of overexpressing the Her-family of proteins is that it presumably contributes to uncontrolled growth signal transduction and hence cellular transformation [106]. However, the exact role of the Her-2/neu receptor expression in the pathogenesis of breast cancer remains unclear. Also, tumors overexpressing Her-2/neu show low responsiveness to adjuvant therapy that includes cyclophosphamide, methotrexate and 5'flourouracil (CMF) [52]. Furthermore, Her-2/neu seems to synergize with the multidrug resistance protein p170mdr-1, rendering breast cancer more resistant to taxol [193]. The cell surface localization of the Her-2/neu makes it a candidate for targeted immunotherapy [15, 135, 138]. Several clinical trial studies are underway utilizing an anti-Her-2/neu monoclonal antibody, tratuzumab (Hereceptin), which has potent antiproliferative activity towards the cancer cells expressing Her-2/neu [125, 67, 68]. It has been demonstrated by several groups that the combination of anti-Her-2/neu treatment and chemotherapy elicits an additive antitumor effect resulting in tumor growth inhibition [189, 120]. Additionally, there is evidence indicating that some patients have existing immunity against Her-2/neu. Several studies have demonstrated the presence of humoral [38] and cellular responses [37] against Her-2/neu. These findings indicate that targeting Her-2/neu could suppress the malignant phenotypes of Her-2/neu-overexpressed tumors and strongly suggests that Her-2/neu could serve as an excellent target for developing anti-cancer immunotherapies specific for Her-2/neu expressing tumors.

3 Self-Tolerance and Induction of Tumor Immunity

The goal of tumor immunologists is to develop immunotherapeutic strategies to generate tumor immunity capable of eliminating cancers. While this goal has been persistently pursued, the road to achieving this goal has not been consistent. The evidence that the immune system in some instances is effective against tumors proves that the development of immunotherapeutic strategies to combat cancer is worth the pursuit. In order to understand the lack of immune responses against cancer and achieve tumor immunity, awareness of the concept of self versus nonself antigens is critical. The principle of the immune system is to tolerate self-antigens but develop vigorous responses against foreign antigens [160, 161]. The discrimination between self and nonself antigens for T-cells occurs in the thymus

where lymphocyte precursors first assemble T-cell receptors (TCR). Following TCR recombination T-cells are selected based on the interaction with peptide/MHC molecules. If T-cell interactions are of low-to-intermediate affinity for MHC/peptide complexes, these T-cells are positively selected and become part of the T-cell repertoire [74, 81]. However, when the interaction of T-cells with self-antigens is too strong, they are negatively selected resulting in the clearance of high avidity T-cells (also called clonal deletion) [148, 163]. Although it is quite likely that central tolerance deletes the bulk of self-reactive T-cells, there is accumulating evidence that self-reactive T-cells that have reached the periphery can also be deleted by peripheral tolerance [14, 132]. Although the identification of TAA encoding mutated cellular genes serves as a target for T-cell immunity, the majority of the currently defined TAA are often mutations in proto-oncogenes and tumor suppressor genes that lead to the development of cancer. Since these TAA are self-antigens and the immune system is trained not to respond to these molecules, mechanisms of self-tolerance dampen the immune responses against TAA. As such, the development of immunotherapeutic strategies against self-tumor antigens is not as simple as previously thought.

Based on transgenic mouse models expressing foreign antigens as self-antigens, it is clear that immune-tolerance is capable of deleting self-reactive high avidity T-cells against the transgene (self), thereby leading to self-tolerance [47, 48]. However, T-cell elimination through tolerance is not absolute, since self-specific T-cells can be isolated from tolerant hosts [75, 58, 25]. A characteristic of these self-reactive T-cells is that the majority of these cells are of low avidity [76, 57, 26]. This raises the question: what is the useful contribution of these low avidity T-cells to the immune defense? The answer to this question probably relates to the degenerate specificity of the TCR. Complete elimination of all T-cells reactive against self-antigens would severely restrict the diversity of the immune repertoire. It is possible that T-cells recognize self-antigens with low avidity and may be able to recognize other antigens with high avidity. Even though TCR are specific for particular epitopes, it is also clear that such TCR can recognize a variety of related ligands [79, 71] and even peptides without apparent homology to the original antigenic peptide [97]. This represents an optimal repertoire that is capable of recognizing a maximum diversity of antigens while being functionally tolerant to self-antigens. Although the T-cell repertoire for self-antigens is severely restricted, a central question is whether the available repertoire of T-cells specific for tumor-self antigens is sufficient in number or avidity to mount an effective anti-tumor response. For example Morgan et al. [101] and de Visser et al. [33] demonstrated that the activation of low-avidity $CD8^+$ T-cells specific for a self-tumor Ag epitope can protect against tumor cell challenges in mice without the induction of an overt autoimmune reaction. Thus, the observation that low-avidity T-cells persist in vivo and that they can induce an antitumor response underscores their potential role in antitumor immunity and offers an important component for the antitumor response. This raises the question of which conditions should be optimized in order to maximize the ability of low avidity T-cells to eliminate tumors.

4 Her-2/neu Transgenic Mice

The availability of strains of transgenic mice expressing tumor antigens has promoted research into the investigation of tissue specific transformation properties of a number of oncogenes [70]. Additionally, these transgenic models could serve as tools for assessing the immune responses against self-tumor antigens and evaluating the immunotherapeutic strategies in preventing and curing tumors. Although the pathogenesis and progression of tumors in these oncogene transgenic mice resembles the human disease, these models have drawbacks. Since the expression of oncogenes is driven by specific promoters the timing of expression of the oncogene does not completely overlap with the human situation. This has important immunological ramifications influencing the timing and intensity of immune-tolerance. Even though these models are not perfect, the lessons learned from them have helped us to better understand: (1) how self-tolerance influences the immune-repertoire, (2) to analyze the immune responses against self-tumor antigens, (3) to evaluate immunotherapeutic strategies to overcome tolerance, and (4) to improve antitumor vaccination strategies.

Almost 20 years ago Muller et al. reported the first Her-2/neu transgenic mice [102, 53]. Today there are several lines of Her-2/neu transgenic mice that overexpress the nontransformed and transformed rat Her-2/neu oncogene. In the nonmutated Her-2/neu transgenic mice, the rat Her-2/neu is under the control of the MMTV promoter. These animals are on the FVB background. Morphological changes are detected in these mice at approximately week 25. Hyperplasia and carcinoma in situ are evident at approximately week 33 followed by invasive lobular carcinoma starting at approximately week 39. Palpable tumors become apparent by approximately week 45. Shortly after the appearance of palpable tumors, animals develop lung and liver metastases that also express elevated levels of neu. This course of disease progression is in marked contrast to the escalated appearance of malignant changes seen in Balb/c transgenic mice expressing the mutated rat neu (BALB-neuT mice) oncogene also driven by the MMTV-promoter [35, 12, 89, 117]. BALB-neuT transgenic mice display one of the most aggressive progressions of Her-2/neu carcinogenesis in which the Her-2/neu is already overexpressed on the surface of the cells of the rudimentary mammary, salivary and Harderian glands on 3–4 week-old mice. At 10 weeks, Her-2/neu positive cells give rise to a widespread atypical mammary hyperplasia, which progresses to form an invasive and metastasizing carcinoma. By week 22–25 palpable masses are detected.

5 Analysis of T-Cell Responses in Her-2/neu Transgenic Mice

A major approach to cancer immunotherapy is the induction of T-cells' responses capable of controlling and killing tumor cells [171]. As explained above the majority of the currently defined TAA are often overexpressed products of normal

cellular genes. Therefore, in practice, these overexpressed proteins pose a significant challenge to the design of effective T-cell immunotherapies due to considerations of self-tolerance. The significance of understanding the mechanism responsible for the persistence of low avidity T-cells relates not only to our understanding of autoimmunity, but also to the potential to target such cells against self-tumor antigens for tumor destruction. Therefore, a central question is whether the available repertoire of T-cells specific for up-regulated tumor-self antigens is sufficient in number or avidity to mount an effective antitumor response. Our group had addressed this fundamental question utilizing the Her-2/neu transgenic mice. In order to evaluate the peptide specific CD8 T-cell responses to neu antigens in FVB-Her-2/neu transgenic mice, we crossed the FVB-Her-2/neu transgenic mice with the A2.1/Kb transgenic mice [183] (called A2xneu mice). The F_1 animals (A2xneu) allowed us, for the first time, to study peptide specific responses in Her-2/neu transgenic mice. In these animals we evaluated A2.1-Her-2/neu responses against the p369–377 and p773–782 peptides that we have identified previously [91]. As a control, A2.1/Kb transgenic mice were crossed with FVB wild type mice (A2xFVB). The application of A2.1-p369 and A2.1-p773 soluble tetramers [192, 124] allowed us to quantitatively and qualitatively analyze the CD8 T-cell specific responses in these animals. Our results indicated that the number and intensity of $CD8^+$/$tetramer^+$ cells derived from A2xFVB mice was significantly higher when compared to A2xneu mice. The CTLs from A2xneu mice required at least 100-fold more peptide to achieve comparable lysis than CTLs from A2xFVB mice [92]. Taken together, the tetramer binding and cytotoxic activity showed that there is a correlation demonstrating a difference in the T-cell affinity between A2xneu and A2xFVB mice for the recognition of neu antigens, indicating that A2xneu mice contain only low affinity T-cells for neu antigens [92]. Later work of Ercolini et al. [40] and Singh and Paterson [153] identified H2q-Her-2/neu specific peptides. Analysis of CD8 T-cell responses in FVB-Her-2/neu transgenic mice revealed that CD8 T-cells are of lower avidity when compared to parental FVB/N mice. These findings further support our data indicating that tolerance is manifested in Her-2/neu transgenic mice by eliminating neu-specific T-cells of high affinity.

6 Strategies for the Induction and Enhancement of Tumor Immunity in Her-2/neu Transgenic Mice

6.1 Peptide Vaccination

Having demonstrated that CTLs from A2xneu (tolerant) mice recognize targe T-cells pulsed with peptides or tumor cell lysates [92, 30], a critical question in tumor immunology is whether the residual T-cell repertoire for tumor-self-antigens would have an antitumor effect. We first evaluated the antitumor effect either by adoptive transfer of peptide-specific CTLs or peptide immunization. Animals were adoptively transferred, once, twice or three times with p369- or p773 CTLs derived

from A2xFVB or A2xneu mice. A single transfer of CTL derived from A2xFVB mice rejected the tumor. In contrast, three transfers of the p369- or p773-CTLs from A2xneu mice inhibited only 40 % of the tumor growth, while less than three CTL transfers demonstrated an even lower efficiency for tumor growth inhibition [92]. We also compared animals that were immunized once, twice or three times with the p369- or p773- peptides. Animals immunized three times with the peptides showed ~20- % tumor growth inhibition, while two immunizations induced a ~12–15 % tumor growth inhibition and one a ~7–9 % tumor growth inhibition. These results indicate that although the residual low affinity repertoire from Her-2/neu mice could be activated these CTLs have a minimal effect in controlling the tumor growth. Studies from other groups indicate as well that peptide vaccination alone is not sufficient or effective in preventing the tumor growth in Her-2/neu mice [111]. These results are similar to those seen in cancer patients where immunizations with Her-2/neu specific-epitopes did not have an effect in controlling the tumor growth [194]. These data suggest that tolerance compromised the immune repertoire against self-tumor antigens hampering the antitumor immune responses.

The identification of immunodominant epitopes is critical for the understanding of the immune responses and it is a prerequisite for the design of specific immunomodulatories therapies. However, as we have demonstrated, a major limitation for the use of an immunodominant epitope from a tumor-self antigen is tolerance. The use of immunodominant peptides such as p369–377 and p773–782 might be optimal for the stimulation of high affinity T-cells, but these peptides suboptimally stimulate the low affinity T-cells. Considering that the interaction of the TCR with the peptide-MHC ligand is highly flexible and that the same TCR can recognize many different epitopes [196, 157, 34, 167], several laboratories have demonstrated that amino acid alterations in T-cell epitopes could enhance stimulation of T-cell populations for the nominal epitope [66, 122, 13]. A major goal in tumor immunization is to circumvent tolerance. Many groups have attempted to enhance the immunogenicity of the peptide by modifying the amino acid sequence to enhance the binding capacity of the peptide to MHC class I molecules [65, 61, 123]. Most of these studies have changed a single amino acid corresponding to a substitution in the anchor motif. For example, substituting a threonine with a methionine at position 210 of the gp:209–217 peptide (heteroclitic peptide) increases the affinity of the peptide fivefold. However, vaccination of patients with this peptide showed no objective clinical response [143]. This raises the question of whether vaccination with heteroclitic peptides of high affinity for a self-antigen best suits the induction of an antitumor response against a self-tumor antigen. Most probably, high affinity heteroclitic peptides are not the best choice as tumor vaccines because the CTL repertoire against these heteroclitic peptides will also be of high avidity, even against the native immunodominant peptide. As such, tolerance might have eliminated the CTL repertoire against the heteroclitic peptides. Therefore, immunizations with the high affinity heteroclitic peptide might not be of clinical value to develop cancer vaccines as demonstrated with vaccinations with the gp: 209–217 peptide. We have taken a different approach to circumvent tolerance against immunodominant epitopes by identifying crossreactive peptides (CP). If we consider that not all possible CP are naturally processed

and presented, the possibility exists that T-cells against these peptides persist in the repertoire and can be used as targets to induce a stronger antitumor response against these immuno-dominant epitopes present on the tumor cells. An important issue becomes which strategy should be used in order to identify the specific amino acid in the epitope that must be altered and with which amino acid it should be substituted. We have used a novel method called positional scanning synthetic peptide combinatorial library (PS-SCL) that allows to identify the most effective residues at each position of the T-cell epitope recognized by a T-cell clone [65, 61, 123]. Based on the screening of PS-SCL we identified potential amino acids that can be substituted in the primary sequences of the p773–782 peptide [94]. Three CP peptides were identified that induce CTL responses of higher affinity in A2xneu mice when compared to the native p773–783 peptide. These CTLs recognize $A2^+$-Her-2/neu^+ tumors with high efficiency. Moreover, multiple immunizations with CP significantly prolonged the survival of tumor bearing A2xneu mice. These results demonstrate an alternative approach where it is possible to circumvent tolerance with the identification of CP and that these peptides could be of significant clinical value. Current studies in our laboratory are optimizing the use of these CP for tumor vaccination.

6.2 Dendritic Cell Vaccination

Dendritic cells (DC) are the most powerful antigen presenting cells that process and present antigens for the stimulation of class I and class II restricted immune responses [6, 100]. The use of DC has become a hallmark for tumor vaccination due to their capacity to regulate T-cell immunity [5, 103]. Mature or activated DCs express a full complement of costimulatory molecules and produce cytokines that are necessary and required for the activation, expansion and maintenance of the immune response [5, 103]. There is a plethora of information in preclinical and clinical studies examining the efficacy of DC vaccination. The major advantage of DC vaccination is that these cells could be pulsed with peptides [4], proteins [144], cell components [82] and viral vectors [59] expressing the antigens present on tumor cells. Our group has compared the antitumor immune responses using DC pulsed with peptides [92], soluble neu protein [30] and apoptotic tumors [30] in FVB-Her-2/neu mice. The advantages in using DC pulsed with proteins or apoptotic cells are that the immune responses are not restricted to single immunodominant epitopes and could stimulate an immune response to numerous antigens activating both $CD4^+$ and $CD8^+$ T-cell responses [5, 103]. Our results indicate that DC-vaccination of Her-2/neu transgenic mice pulsed with soluble neu protein and apoptotic tumors induce a stronger immune response when compared to DC-peptide vaccination resulting in a significant delay of tumor growth [30]. Sakai et al. [142] demonstrated that the DC pulsed with recombinant adenovirus expressing truncated neu protein induced a specific anti-neu antibody and T-cell response that delayed the onset of mammary carcinomas in BALB-neu T mice. Recently, Chan et al. [18] compared the immune and antitumor responses between adenovirus (AdVneu)-transfected DC and plasmid DNA expressing neu in Her-2/neu

transgenic. They demonstrated that modified DC vaccine is more potent than DNA vaccine in both protective and preventive animal tumor models.

6.3 Addition of Costimulation

It is generally accepted that T-cell activation requires two signals [86, 182], the first provided by the TCR and peptide/ major histocompatibility complex interaction (MHC) and the second from the antigen-independent interaction between T-cell-expressed CD28 molecule and the costimulatory B7.1 (CD80) and/or B7.2 (CD86) ligands, expressed on the surface of APCs. There is growing evidence that the two-signal model is an oversimplification of the mechanism for the activation of an immune response and that the signaling of other accessory molecules (third signal) might be necessary and important to amplify and effectively expand the immune response. Among these other accessory molecules, the TNF receptor family, including CD27, CD30, CD40, 4-1BB, and OX40 have gained importance as co-stimulatory molecules delivering signals that prolong and propagate T-cell responses [28, 185]. It has been shown that antibodies against OX-40/4-1BB have mitogenic signals for T-cell activation and growth [151, 49], induce a vast amplification of T-cell mediated immune responses [85, 186], inhibit apoptotic cell death [164, 84] and stimulate long-lived T-cell responses [51, 116]. Furthermore, administration of monoclonal antibodies against 4-1BB or OX40 as a single agent induces immune responses that significantly reduce the growth of tumor [80, 168]. We evaluated whether immunization with a combination of DC pulsed with apoptotic tumor cells and anti-OX40/anti-4-1BB mAbs would improve the immunotherapeutic efficacy in FVB-Her-2/neu mice. We tested the immunization of DC with each of the antibodies alone or with the combination of both anti-OX40 and anti-4-1BB mAbs. The rationale for simultaneously using anti-OX40 and anti-4-1BB was that anti-OX40 predominantly interacts with CD4 T-cells [29], while anti-4-1-BB predominantly interacts with CD8 T-cells, therefore, it might be possible to amplify both T-cell subsets resulting in a more effective antitumor response. Our results indicated that DC-immunization plus anti-OX40 or anti-4-1BB mAb further enhanced the antitumor response when compared to animals that received DC-immunization alone [29, 92, 30]. Interestingly, DC-immunization plus anti-OX40/anti-4-1BB mAb significantly improved the T-cells responses resulting in a ~70% tumor growth inhibition [30]. Murata et al. [104] also demonstrated that anti-OX40 mAb enhanced the antitumor effect of Ag-specific GM-CSF-secreting vaccine in FVB-Her-2/neu mice.

6.4 Cytokines

A number of studies have revealed the relationship between tumor cells, inflammatory cells and cytokines [22, 166]. Cytokines within the tumor can contribute to the progression of tumors or play a role in controlling tumor growth [50, 136]. With the

use of cytokines the environment of the hosts' tumor could be altered to favor or facilitate tumor immunity [60]. Dr. Guido Forni pioneered the use of cytokines for the treatment of tumors in Her-2/neu mice and his group demonstrated that administration of recombinant IL-12 slowed the progression of spontaneous mammary carcinomas in FVB-Her-2/neu and BALB-neuT mice [11, 23, 36]. The injections of IL-12 were associated with inhibition of angiogenesis, infiltration of reactive cells, production of proinflammatory cytokines, and activation of inducible nitric oxide synthase (iNOS). Following this seminal study they optimized the use of IL-12 for the induction of stronger antitumor immune responses and combined it with DNA-vaccination or allogenic vaccines [109, 118] as described below. Elizabeth Jaffee's group had evaluated the use of GM-CSF on tumor modified cell vaccines for the induction of antitumor responses in Her-2/neu transgenic mice [133, 187]. These studies are also discussed below.

6.5 DNA Immunization

The ability of DNA vectors to activate cellular and humoral immune responses has prompted intensive study into their use for vaccine development and as an immunotherapeutic modality. As such, DNA vaccines have emerged as a potentially important form of vaccination in the control of cancers [88]. The efficacy of DNA vaccines has been demonstrated in animal tumor models by targeting tumor-associated or tumor-specific antigens [172, 170]. The advantage of DNA vaccination is that it is a cell free system. Injection of plasmid-DNA into skin or muscle results in plasmid DNA uptake by APCs at the site of plasmid injection which subsequently induce humoral and cellular responses. The immunogenicity of the DNA-vaccines has been enhanced with the use of adjuvants (e.g., alum, cytokines, or LPS) or the incorporation of unmethylated CpG motifs that help skew the immune response to a Th1 type response [175]. Many groups have evaluated intramuscular DNA-plasmid vaccination for controlling tumor growth in Her-2/neu mice [20, 3, 139, 121, 128, 140]. Most of these studies used a plasmid expressing the neu gene and demonstrated that intramuscular vaccination with DNA-neu-plasmid could induce cellular and humoral responses resulting in tumor growth inhibition [20, 3, 139, 121, 128, 140]. Capello et al. [16] demonstrated that DNA-vaccination could be enhanced in BALB-neuT mice if costimulation was provided with the administration of soluble mouse LAG-3 (lymphocyte activation gene-3/CD223). Quaglino et al. [130] showed that vaccination of DNA-plasmid by electroporation at 10 week intervals provided a greater and more persistent immune response and kept all 1-year-old BALB-neuT mice free of tumors. These results indicate that repeated courses of immunizations are required and that in vivo electroporation enhances DNA-vaccination. Additionally, several groups have demonstrated that xenogeneic vaccination with DNA induced an immune response in BALB-neuT mice [129]. Recently Smorlesi et al. [158] compared intradermic injection, gene gun delivery and intramuscular

injection of DNA vaccine alone or with electroporation. They concluded that the vaccine delivery methods analyzed elicited diverse immune mechanisms that differently prevented the appearance and the development of spontaneous mammary carcinomas. In their hands the use of intramuscular injection plus electroporation resulted in the best antitumoral effect and in the generation of a Th1-type immune response. The combination of DNA vaccine electroporation with systemic IL-12 administration, effectively prevents the onset of carcinomas in most BALB-neuT/p53^{172R-H} mice [118].

6.6 Bacterial Vectors

Live attenuated mutants of several pathogenic bacteria have been exploited as potential vaccine vectors for antigen delivery [31]. Attenuated invasive human bacteria, such as Listeria, Salmonella and Shigella, have been used as plasmid DNA vaccine carriers and their potency has been evaluated in several animal models. This delivery system allows the administration of DNA vaccines together with associated bacterial immunostimulators directly to professional antigen presenting cells. Yvonne Paterson pioneered the use of Listeria monocytogenes for vaccination purposes and data from her group demonstrated that the delivery of this bacterium expressing truncated forms of the neu oncoprotein increased the immunogenicity of this self-antigen, induced CTL responses and they could identify new T-cell epitopes [152, 154, 155]. This group, further demonstrated that immunization of Listeria monocytogenes expressing Her-2/neu slowed the growth of implanted tumors in FVB-Her-2/neu transgenic mice.

6.7 Modified Tumor Cells

There is evidence indicating that tumor cells are capable of directly and indirectly activating tumor-specific T lymphocytes [24]. Indirect activation of immune responses by tumor cells involves crosspriming of tumor antigens by APCs inducing the activation of T-cell responses. In contrast, direct activation of immune responses by tumor cells is facilitated when tumors are able to present tumor antigens and directly activate T-cell responses. These responses are greatly enhanced when tumor cells are genetically engineered to express costimulatory ligands, (e.g., CD80), secrete cytokines (e.g., IL2, IL-12, GM-CSF) or used as allogeneic vaccines. These modified tumor cells are capable of inducing immune responses in naïve animals that could protect against challenge with unmodified tumor cells [83]. Several groups have used genetically modified tumor cells to evaluate their efficacy in inducing an antitumor response in Her-2/neu transgenic mice.

6.7.1 Modified Cells Expressing Neu and GM-CSF

Reilly et al. [133] modified NIH-3T3 cells to express neu and GM-CSF. Their study indicated that immunization with 3T3-*neu*/GM-CSF cells induced antibody and T-cell responses in FVB-Her-2/neu mice. In a prophylactic setting immunization with 3T3-*neu*/GM-CSF cells significantly delayed the transplantable tumor or delayed the onset of spontaneous tumors in these mice. In subsequent studies, the same group demonstrated the importance of both humoral and cellular responses for tumor eradication [134]. Animals receiving the combination of neu-specific-CTLs and neu-specific-IgG were fully protected, while animals receiving only CTL or antibody therapy were partially protected.

6.7.2 Modified Allogeneic Tumor Cells Expressing IL-2, IL-12, IL-15, or INF-γ

De Giovanni et al. [32] expressed the genes for IL-2, IL-12, IL-15 or INF-γ on a tumor cell derived from FVB-neu transgenic mice (N202.1A, $H2^q$ haplotype). Immunization of BALB-neuT mice ($H2^d$ haplotype) with these modified cells demonstrated that those cells secreting IL-12 had the most powerful immunopreventive activity. More than 80% of BALB-neuT mice were tumor free for 1-year. Cells lacking Her-2/neu or allogeneic antigens failed to induce an antitumor response. The immune response was dependent on the production of INF-γ and the induction of an antibody response.

6.8 Viral Vectors

Recombinant viral vaccines have been used in the development of cancer vaccines for the past 10 years. The advantage of viral vectors is that it offers the ability to express single or multiple tumor antigens [159] along with an array of immune costimulatory molecules [46] or immune-enhancing factors [56]. Additionally viral vectors could provide a danger signal enhancing the immune responses [191]. There are different viral vectors such as the vaccinia virus, adenovirus, canarypox virus, fowlpox virus, alphavirus, etc, that have been modified and used in preclinical and clinical studies [55, 188]. Each viral vector has advantages and disadvantages like immunogenicity, existing immunity, safety, manufacturing, loading gene(s) of interest, efficacy of gene expression, etc. One of the potential ways in which cancer vaccines can be optimized is through the use of different viral vectors to deliver the same tumor antigen (heterologous prime-boost) [55, 188]. Several laboratories have evaluated the use of viral vectors to induce antitumor immune responses in Her-2/neu transgenic mice. Schwaninger et al. [145] show that virosomes, which consist of reconstituted viral envelopes without viral genetic material, can act as a carrier and an adjuvant for the induction of humoral and cytotoxic immune responses

delaying tumor formation in Her-2/neu transgenic mice. Several other groups have shown that recombinant adenovirus expressing different forms of the neu oncoprotein stimulated the production of specific anti-neu antibodies, T-cell responses and prevented or delayed the onset of mammary carcinomas in the BALB-neuT mice [142, 44, 119, 18, 45]. Tegerstedt et al. [169] demonstrated that murine polyomavirus (MPyV) VP1 virus-like particles (VLPs) containing the extracellular and transmembrane domain of Her-2/neu also delayed onset of mammary tumors in BALB-neuT mice.

6.9 Nanoparticles

Nanotechnology, is defined as the biomedical application of nanosized systems which measure 1–1000 nm [17]. Key advantages of many nanoparticles are their low toxic effects and biocompatibility. Nanoparticles are considered to have the potential as novel cellular probes for both diagnostic (imaging) [184] and therapeutic purposes (drug/gene delivery) [78]. Drug targeting by nanoparticles or nanocapsules offers enormous advantages as it reduces the quantities of the required drugs, the pharmaceutical effects are achieved, and it also minimizes side-effects; protects drugs against degradation and enhances drug stability. The group of Dr. Nejat Egilmez explored the use of nanoparticles or microspheres for the induction of tumor immunity in FVB-Her-2/neu transgenic mice. They generated biodegradable microspheres containing IL-12 and GM-CSF. Intratumoral (i.t.) injection of these microspheres promotes the suppression of established primary tumors, the development of systemic antitumor immunity, and the complete eradication of disseminated micrometastatic disease in a transplantable tumor model [107, 108]. However, tumor regression was found to be temporary since recurrence of tumors were observed.

6.10 Toll Like Receptor Ligands

The innate immune response relies on the recognition of the antigen by receptors that recognize specific structures found exclusively in microbial pathogens termed pathogen-associated molecular patterns (PAMPs) [8]. Recent studies have demonstrated that recognition of PAMPs by APCs is mediated by a Toll-like receptor (TLR) family [98, 73]. There are currently more than 10 known TLR family members capable of sensing bacterial wall components, such as LPS (TLR-2/4), CpG-DNA (TLR-9), flagellin (TLR-5), as well as other microbial products [165]. Recognition of PAMPs by TLRs triggers maturation and activation of APCs that includes upregulation of MHC and co-stimulatory molecules, and secretion of proinflammatory cytokines and chemokines. This maturation of APCs significantly increases their ability to prime naïve T-cells. In this way, TLRs link the recognition of pathogens with induction of adaptive response. Now that specific ligands have

been identified for most of the TLRs, it is finally possible for immunotherapy to move away from the nonspecific effects of whole bacterial extracts and determine whether the same or even better therapeutic responses may be induced using synthetic TLR ligands. Many studies had demonstrated that injections of TLR-ligands could significantly improve vaccination formulation [173, 177]. Sfondrini et al. [147] showed that systemic treatment of CpG-ODN reduced lung metastases induced by transplantable tumor in Her-2/neu mice. Recently Nava-Parada et al. [111] demonstratedthat peptide vaccination given in combination with CpG-ODN was effective in inducing CTL responses with antitumor activity in BALB-neuT mice. Our group has compared the antitumor effect of different TLR-ligands such as: Poly I:C, LPS, flagellin, imiquimod and CpG-ODN in BALB-neuT mice. Only (i.t.) injections of CpG-ODN induced the rejection of primary tumors in ~30 % of the BALB-neuT (Sharma et al. submitted for publication). Animals that did not reject the tumor significantly delayed the tumor growth. In order to target the CpG-ODN at the tumor site, we chemically conjugated an anti-Her-2/neu mAb with CpG-ODN. Treatment with anti-neu-CpG-ODN induced the rejection of tumors in BALB-neuT mice. These results indicate that CpG-ODN-targeted therapy could be used as a novel strategy for the induction of antitumor responses.

6.11 Heat Shock Proteins

Heat shock proteins (HSPs) help to maintain cell homeostasis under physiological and stress conditions, however, some HSPs are potent inducers of immunity and have been used as vaccine adjuvants to enhance immune responses [9]. HSPs are potent inducers of innate and antigen-specific immunity. They activate DC partly through toll-like receptors, increase antigen presentation resulting in the activation of T-cell and humoral immune responses [72]. Manjili et al. [96] prepared aheat shock complex of HSP110 with the intracellular domain of human HER-2/*neu* and demonstrated that this complex induced IFN-γ $CD8^+$ producing T-cells capable of delaying the onset of spontaneous mammary tumors on FVB-Her-2/neu transgenic mice.

6.12 Depletion of Immunosuppressor Cells

There is accumulating evidence indicating that antitumor immune responses could be suppressed or inhibited by the presence of immuno-suppressor cells [131]. The most characterized suppressor cells are the $CD4^+CD25^+$ T-cells or Tregs [141]. The depletion of $CD4^+CD25^+$ T-cells by the administration of anti-CD25 mAb has been shown to suppress the growth of a variety of different syngeneic tumors in mice [114]. The observation that the removal of immunoregulatory $CD4^+CD25^+$ T-cells can abrogate unresponsiveness to syngeneic tumors in vivo, leading to the

spontaneous development of tumor specific responses, indicates that the maintenance of self-tolerance against tumor-self antigens could potentially be lifted. Several groups including our own have evaluated the role of T-regs in regulating the immune responses in Her-2/neu mice. Ercolini et al. [41] show that pretreatment with cyclophosphamide which inhibits T-regs allowed the activation of high avidity T-cells in Her-2/neu mice. The combination of cyclophosphamide chemotherapy with Her-2/neu-specific vaccination results in a stronger antitumor response when compared to vaccination alone. Depletion of T-regs by repeated administrations of anti-CD25 mAb prolonged the survival, reduced carcinoma multiplicity and induced an antibody and CTL-mediated reactivity against Her-2/neu in BALB-neuT mice [2]. In another study, the combination of peptide vaccination and anti-CD25 mAb treatment significantly enhanced the CTL responses and a single vaccination of peptide+CpG-ODN given after three daily injections of anti-CD25 mAb completely prevented the occurrence of spontaneous tumors in BALB-neuT mice up to 35 weeks of age [111]. Our results indicate that the number of T-regs keep accumulating over time at the tumor site. We have evaluated the effect of blocking the suppressive activity of T-regs in a therapeutic setting with our CpG-ODN vaccination strategy. BALB-neuT tumor bearing mice treated with i.t. injection of CpG-ODN or anti-neu-CpG-ODN plus injections of anti-GITR mAb resulted in the complete rejection of the primary tumor and induced a long term memory response (Sharma et al. submitted for publication).

Although T-regs are well known as suppressor cells there are other type of suppressor cells like myeloid suppressor cells (MSC, also know as immature myeloid cells, IMC or M2- macrophages). The MSC are characterized by the expression of $CD11b^+$ $Gr1^+$ surface markers. MSC can suppress the activation of $CD4^+$ and $CD8^+$ T-cells inhibiting the generation of antitumor responses [146]. MSC are though to be induced by a variety of cytokines and growth factors (TGF-β, VEGF) which are produced within the tumor microenvironment. MSC have poor antigen-presenting capability, produce factors that suppress T-cell prol-iferation and activity, and promote angiogenesis [146]. Melani et al. [99] found a direct correlation between tumor multiplicity and increased proportion of $CD11b^+$ $Gr1^+$ cells in BALB-neuT mice. Ambrosiano et al. [2] demonstrated that the reduction of T-regs correlate with the disappearance of $CD11b^+$ $Gr1^+$ cells in BALB-neuT mice. Taken together, these results indicate that immunosuppressor cells heavily influence the immune responses in Her-2/neu transgenic mice.

6.13 Combination of Immunotherapy with Antiangiogenic or Chemotherapy

A number of preclinical and clinical cancer studies demonstrate an increase in antitumor efficacy when combining more that one treatment approach [62]. Significant benefits for combinations such as radiation and cytokine therapy [105], radiation and biologically targeted agents (antiangiogenic agents, anti-EGFR antibodies) [43], and

tyrosine kinase inhibitors and immunotherapy [112] have been shown. For example, in Her-2/neu patients the combination of paclitaxel and Hereceptin produced higher response rates and longer survival duration than each therapy alone [87]. These data support the concept of combination therapies being generally superior to monotherapies. Above we illustrated several immunotherapeutic strategies to induce antitumor responses. However, the majority of immunotherapies alone are not sufficient to eradicate the tumor in Her-2/neu transgenic mice. Angiogenesis is the ability of preexisting vasculature to send out capillary sprouts leading to the formation of new vasculature [54]. It is now a well-accepted idea that progression of solid tumors is intrinsically dependent on angiogenesis for growth of the primary tumor and metastatic lesions. To inhibit tumor angiogenesis, we produced a soluble form of the Flt-1 (sFlt) molecule secreted by tumor cells in order to block the biological activity of the VEGF. Treatment with sFlt, delayed the tumor growth. We tested the combination of immunotherapy and antiangiogenic therapy and our results showed that the combination of these therapies eradicated tumors in Her-2/neu mice with a small tumor burden [29]. Animals with a larger tumor burden and treated with immunotherapy and antitangiogenic therapy resulted in a 90 % inhibition of tumor growth [29]. Holmgren et al. [63] show that DNA vaccinations encoding for angiomotin and Her-2/neu, inhibited angiogenesis and induced anti-neu responses in which 80% of the BALB-neuT mice were tumor free for more than 70 weeks.

Several groups have demonstrated that the application of chemotherapy at low doses increase the potency of immune-mediated cytoxicity or tumor vaccines [21]. Machielis et al. [95] showed thatcyclophosphamide, paclitaxel, and doxorubicin, when given in a defined sequence with modified cells secreting GM-CSF and expressing Her-2/neu enhanced the potency of the vaccine and amplified the T helper 1 response thereby, delaying the tumor growth in FVB-Her-2/neu mice. The combination of IL-12 and tamoxifen controlled the tumor growth and 80 % of the Her-2/neu mice were tumor free [110].

Above we summarized most of the strategies used to evaluate immune and antitumor responses in Her-2/neu mice. The knowledge acquired, so far, through the use of the different vaccination strategies allows us a better understanding of the requirements for the generation of an immune response against Her-2/neu. Future advances in the understanding of the mechanisms for action of the immune responses against self-tumor antigens will permit the enhancement or development of new vaccination strategies.

7 Analysis of Immune Responses Against Self-Antigens in Aging Tumor Model

Cancer statistics show a disproportionately higher burden of tumors in the older population [190]. Furthermore, the numbers of older people diagnosed with cancer is expected to increase since the average life span within the elderly population has

increased. Even though many laboratories are evaluating a variety of vaccination strategies to induce antitumor immune responses, none of these laboratories has taken into consideration the effect that aging has on the anti-tumor immune response. Although most murine models used for cancer research use young mice, cancer is primarily a disease of aging individuals. In the US > 50 % of cancer diagnoses are made after the age of 65 years. Since the immune system of the aged is different to that of the young, and is in a state of hypo-responsiveness, the conclusions drawn from studies on young animals cannot be extrapolated to represent the events taking placed in aged individuals. We have demonstrated that immunotherapeutic intervention could be effective in young animals, but that the same therapy is not effective in old animals [93]. Our group has previously shown that Balb/c animals are successful in eradicating tumor cells expressing Enhanced Green Fluorescent protein (EGFP) as a surrogate tumor antigen. These young animals were protected against subsequent challenges with either the EGFP-modified or the wild-type tumor. In contrast, aged Balb/c mice did not mount a protective response to immunization with EGFP-cells. Long term memory responses against wild type tumors were only developed in these old mice when EGFP-CD80 expressing tumor cells were administered in combination with anti-OX40 and anti-41BB mAb [149]. These data indicate that it is possible to convert the immune repertoire in the aged animals from a nonresponder to a responder status with the inclusion of additional costimulation. In the presence of anti-OX40 or anti-4-1BB mAb, T-cells responses were similar in old and young mice [190].

Our group recently demonstrated that old Balb/c mice contained twice the amount of $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ populations in spleen and lymph nodes when compared to spleens and lymph nodes from young mice [150]. Depletion of $CD25^+$ cells with anti-CD25 mAb in old mice resulted in the rejection of BM-185-EGFP tumor cells, resulted in the generation of a protective memory response against BM-185-wild type cells and restored antitumor T-cell cytotoxic activity [150]. These results indicate that a direct correlation between the expansion of T-regs and immune deficiency exists in the old and the depletion or reduction of T-regs might be critical to optimally activate an immune response in the aged. Taken together, these results have important implications for the development of vaccination strategies in the elderly indicating that the aged T-cell repertoire can be exploited for the induction of tumor immunity and that additional coactivation and or inhibition of the suppressive activity of T-regs might be required for an optimal antitumor immune response in aged hosts.

We have recently evaluated whether targeting APCs following injection of TLR-ligands such as Poly I:C, LPS, flagellin, imiquimod and CpG-ODN would induce the antitumor responses in the old. Our results indicated that only injections of CpG-ODN completely rejected the tumor in both young and old mice. Injections of Poly I:C also induced the rejection of tumors in the young but not in the old. Treatment with injections of LPS, Imiquimod or flagellin did not have any effect in controlling the tumor growth in young or old mice (Sharma et al. In Press). These results indicate that not all TLR-ligands are able to induce an antitumor response and that there are differences among the various TLR-ligands in their capacity to induce an

antitumor response in old mice. This information is very important for the selection of adjuvants in order to induce or enhance an immune response in the elderly.

Even though the data presented above and the evaluation of other antitumor responses by several laboratories using different tumor models [127] are very encouraging and many lessons can be learned about the behavior of the antitumor immune responses in the elderly, through such models, we have to remind ourselves that in general the majority of these tumor models rely on immunogenic tumors. As such, it will be more difficult to translate the results from these immunogenic tumor models into a clinical setting for the treatment of tumors in the old. As described in several chapters of this book, the T-cell component of the aged immune system is dramatically compromised, i.e., the immune response is impaired and the repertoire is constricted. To date very little data exists on the immune responses against self-tumor antigens in the aging population. So far there are no reports evaluating antitumor immune responses in aged tumor models where tolerance and spontaneous tumor progression are present simultaneously. The effect of aging on T-cell tolerance remains to be elucidated. Therefore, it is clear that there is a need for relevant animal tumor models which include aspects of self-tolerance and development of spontaneous primary and metastatic tumors in the elderly. Models like this are critical for the development and optimization of more accurate cancer-related immunotherapeutic strategies for the elderly. We have observed that one of the consequences of crossing FVB-Her-2/neu mice with HLA-A2 mice (A2xneu) is that spontaneous tumors appear in these animals when they are 22–27 months old. Therefore, the A2xneu mouse model represents a unique model where aging, tolerance and spontaneous tumor progression are present simultaneously. The A2xneu mouse model closely reflects the human disease, where the testing of immune responses, vaccination or immunological strategies against self-tumor antigens will have a higher chance of being relevant in the human situation. There are several reasons as to why there is a lack of more studies evaluating antitumor responses in old mice and one critical factor is the extended time period which is necessary to age these mice and the costs incurred towards this aging. To evaluate antitumor responses in this aged animal model, we have utilized cell lines derived from spontaneous tumors to facilitate the rapid evaluation of the immune and antitumor responses.

The advantage of A2xneu mouse model is that recapitulates the clinical progression and pathogenesis of the human disease. Additionally, the immunological aspects of the A2xneu mouse model closely reflect those of cancer patients whose immune systems are not fully competent to reject their tumors. To test the antitumor responses, we have developed a tumor model utilizing a tumor cell line derived from spontaneous tumors (N202.A2 cell line) that facilitates the rapid evaluation of the antitumor responses. We have already started to evaluate the immune and antitumor responses in old A2xFVB mice and A2xneu mice. Previously we demonstrated that N202.A2 cells grow in A2xneu mice as a consequence of immune-tolerance, but are rejected by young A2xFVB mice. We evaluated whether aging has an effect on the immune system preventing the rejection of N202.A2 cells in old animals. Young (2 months old) and old (18 months old) A2xFVB mice were implanted s.c. with 10^6 N202.A2 cells and tumor growth was evaluated. As expected, young

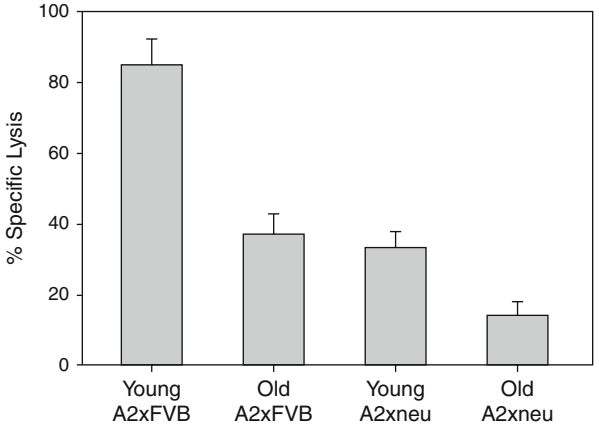

Fig. 1 Analysis of the T-cell responses against the p773-peptide in young and old A2xneu and A2xFVB mice

A2xFVB mice rejected the tumor; however, the tumor grew in old mice. N202.A2 cells formed tumors in young and old A2xneu mice as expected. We also tested whether there was a difference in the priming ability of young and old A2xneu and A2xFVB mice to induce a CTL response after immunization with the p773 peptide. We observed that the CTL activity from young A2xFVB or A2xneu mice was stronger when compared with the CTL activity of A2xFVB or A2xneu old mice (Fig. 1). Interestingly, the CTL from old A2xFVB (non tolerant) mice had a similar cytotoxic activity to the CTL from young A2xneu (tolerant) mice. A very weak CTL activity was detected in old A2xneu mice (Fig. 1). Taken together, these results further support the plethora of evidence indicating that aging suppresses the immune system and that old mice do not have the same capacity to prime a T-cell response as young mice. In agreement with our previous report, old A2xFVB and A2xneu mice have higher numbers of T-regs when compared to young A2xFVB and A2xneu mice. We are continuing with our investigations to determine whether the attenuated CTL responses observed in A2xneu old mice are due to T-reg mediated suppression.

Our results suggest that i.t. injections of CpG-ODN could rescue the immune responses in the old and promote antitumor responses in Her-2/neu mice. We tested the effect of injection of CpG-ODN in old A2xFBV and A2xneu mice. Our results indicate that old A2xFVB mice rejected the N202.1A tumors and old A2xneu mice significantly delayed the tumor growth prolonging the survival of the animals (Dominguez et al. submitted for publication). These results demonstrate that antitumor responses could be promoted in old tolerant hosts. Through the A2xneu mouse model we have successfully uncovered some of the cellular basis for the decline in immune function in the elderly and have begun to elucidate, the conditions and strategies needed to augment the antitumor activity of the aged.

The A2xneu mice represent the first animal model through which it is now possible to evaluate the antitumor immune responses in both old and self-antigen tolerant hosts. This model is invaluable and is of great importance because the results derived from it will allow us to optimize antitumor immune responses in the old. Our group is currently evaluating the immune responses and the strategies to further

enhance the antitumor responses in A2xneu mice. The A2xneu mouse model will enable us to uncover some of the cellular basis for the decline in immune function in the elderly and determine conditions and strategies to augment the antitumor activity against self-tumor antigens in the aged. The information generated from these animals will more comparable to the aging environment and could be better translated for the treatment of cancer in the old.

8 Conclusions

The use of mouse model like the Her-2/neu transgenic mice have provided valuable information to evaluate and establish basic paradigms of tumor immunology since they offer the in vivo environment that cannot be reproduced in vitro [115]. Although tumor animal models like the Her-2/neu transgenic are not perfect, they closely resemble the human situation where it is possible to evaluate the effect of self-tolerance on the immune system and develop strategies for inducing tumor immunity against a self-tumor antigen. As described in this chapter many groups including ours have developed and evaluated different immunological strategies to control the tumor growth in Her-2/neu transgenic mice. Although we might be able to develop immunotherapeutic protocols that are effective in controlling the tumor growth in young Her-2/neu mice, we have to make sure that the same protocols are also effective in old animals since the immune system of the aged is associated with a dramatic reduction in responsiveness as well as functional dysregulation. Additionally, there is strong evidence indicating that the immune interventions applied for the induction of tumor immunity in the young will not be effective in the old. Therefore, the development of animal tumor models where aging and tolerance are present at the same time are critical. Only models like this will allow the optimization of vaccination strategies to effectively stimulate tumor immune responses in both the young and the old. Our A2xneu mouse model provides a unique opportunity to evaluate immune and antitumor responses against a self-tumor antigen where aging and tolerance are present at the same time. Hopefully models like the A2xneu mice will provide valuable information to customize and optimize vaccination strategies that would be effective in both the young and the old. Then, the final challenge is to translate the results of these preclinical models into the clinical setting.

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Cancer Immunotherapy and Aging: Lessons From the Mouse

Claudia Gravekamp

Contents

Abbreviations

APC	antigen presenting cells
CD	cluster differentiation
CEA	carcinoembryonic antigen
COX	cyclooxygenase
CTL	cytotoxic T lymphocytes
CTLA	cytotoxic T lymphocyte antigen
DC	dendritic cells
EGFP	enhanced green fluorescent protein
ELAM	epidermal lymphocyte adhesion molecules
FOXP3	transcription factor forkhead box P3
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA	human leucocyte antigens
HPV	human papilloma virus
ICAM	inter cellular adhesion molecules
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
MMTV	mouse mammary tumor virus
NK	natural killer
PBL	peripheral blood lymphocytes

C. Gravekamp (✉)
Albert Einstein College of Medicine
Department of Microbiology and Immunology
1300 Morris Park Avenue
Forchheimer building, Room 407A
Bronx, NY 10461

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PBMC	peripheral blood mononuclear cells
PGE	prostaglandine E
PPD	purified protein derivate
PRR	pattern recognition receptors
PSA	prostate cancer antigen
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigens
TCR	T cell receptor
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
Thelp	helper T cells
	Tregs, regulatory T cells
VCAM	vascular cellular adhesion molecules.

Abstract: Cancer is a disease of the elderly. Since demographic trends indicate that over the next decades the number of elderly people will increase substantially, strategies for cancer prevention and therapy need to be optimized to older patients. Immunotherapy, either through passive or active immunization is a highly targeted type of therapy that is potentially less toxic than chemotherapy or radiation and could, therefore, be especially effective in older, more frail cancer patients. In particular active immunization, i.e., employing patient's own immune system through vaccination, offers great promise since it can potentially keep cancer permanently at bay. However, it has been shown that older individuals do not respond to vaccine therapy as well as younger adults. This has been attributed to diminished T-cell responses, a phenomenon also observed in cancer patients per se. To develop cancer vaccines that are effective at older age, the availability of preclinical models that can predict age effects on cancer vaccination is critically important. In this review, current knowledge of diminished T-cell responses in cancer patients and elderly, the results of cancer vaccination in preclinical models and human clinical trials and the impact of aging on immunotherapy will be discussed. Finally, experimental approaches will be proposed how to make cancer vaccines more effective at older age.

Keywords: Cancer • vaccines • Immunosenescence • Immunotherapy • Aging • Mouse • tumor models

1 Introduction

Cancer immunotherapy is the manipulation of the immune system against tumor cells. This could be manipulation of the patient's own immune system by active immunization, or the use of humanized antibodies or T-cells activated ex vivo, called passive immunization. So far, promising results have been obtained with passive immunizations using monoclonal antibodies directed against growth factor receptors on tumor cells, or coupled with a radioisotope or a toxin. However, these therapies often develop

side effects such as cardiotoxicity, pulmonary complications, and hematological toxicity (for a review see Klastersky, 2006). Passive immunization with antigen-specific T-cells that have been stimulated ex vivo, so far showed marginal success, is technically very difficult, time-consuming and labor-intensive (for a review see Xue et al., 2005). Adoptive transfer of T-cells that have been genetically manipulated by introducing high avidity T-cell receptor (TCR) in cytotoxic T lymphocytes (CTL) that recognize tumor-specific antigens with much higher efficacy than the patient's own T-cells, seems more promising because they develop long-lasting therapeutic effects, and triggers the production of lymphokines (Xue et al., 2005). Phase I and II human clinical trials are ongoing but results are not available yet. In contrast to passive immunization, active immunization or vaccination can be used preventively and therapeutically. Active immunization against proteins expressed by tumors, called tumor-associated antigens (TAA), can induce long-lasting memory T-cell responses, while passive immunizations have limited duration. Active immunization has less side effects, is technically less difficult to apply, and is less expensive than passive immunizations. Overall, active immunizations are potentially more promising. They are the subject of my own research and the main focus of this review.

2 Cancer Vaccines: The Puzzle and the Promise

The first attempts to develop cancer vaccines on the basis of irradiated tumor cells were unsuccessful (for a review see Schreiber et al., 1998). Tumor cells are poor APC due to the low expression of MHC and costimulatory molecules and poor processing of antigens and presentation of TAA on the membrane. Several decades of research into the presence of TAA have shown that many tumors express antigens that are not expressed in normal adult tissues such as MAGE, GAGE, BAGE, LAGE, NY-ESO-1, or overexpressed antigens that are present at low levels in normal tissues such as CEA, HER2/neu, MUC1, Survivin, or show altered expression by mutation in cellular genes such as MUM1, cdk4, β-catenin (for a review see Gravekamp, 2001). New generations of TAA-based cancer vaccines have become available that are much more powerful in activating the immune system with less severe side effects than irradiated tumor cells. This new generation of vaccines is able to activate different T-cells depending on processing of exogenous or endogenous proteins produced by the vaccine, and subsequent presentation of the antigen (TAA peptides) by antigen-presenting cells (APC) to the immune system. The conventional dogma is that endogenous proteins, for instance delivered into the cytoplasm of an APC by a DNA vaccine, are processed by cytoplasmic enzymes resulting in small peptides, then transported to the endoplasmic reticulum, where peptides can associate with newly synthesized major histocompatibility complex (MHC) class I molecules. These peptide/MHC class I complexes migrate to the membrane of the APC for presentation to the immune system and for subsequent activation of naive CTL. Exogenous proteins, for instance from purified protein or conjugate vaccines, are internalized by APC via endocytosis to an endosomal compartment, where they are digested into peptides

and associated with MHC class II molecules. These peptide/MHC class II complexes migrate to the membrane of the APC for presentation to the immune system and for subsequent activation of naïve T helper cells (T_{help}). Recently, this dogma about processing of exogenous and endogenous proteins has been changed. It has been shown by several research groups (for a review see Cohen et al., 1998) that exogenously produced proteins can be taken up by APC and then presented in the context of MHC class I molecules. Below, several cancer vaccines will be discussed that are particularly powerful in the induction of CTL responses and therefore potentially useful in the development of cancer vaccines for the elderly.

2.1 Peptide-Based Vaccines

Use of peptide-based vaccines is an approach to initiate TAA-specific CTL responses. Such vaccines obviate the need to digest proteins into peptides, a process that is often impaired in tumor cells. Peptide-based vaccines consist of dendritic cells (DC; isolated from the cancer patients themselves) loaded with synthetic peptides derived from TAA that are expressed, but inadequately presented by the tumor. These peptides assemble with MHC molecules that are highly expressed at the cell membrane of DC. Injection of these peptide-loaded DC into cancer patients leads to presentation of TAA-peptide/self-MHC complex to the immune system, activating TAA-specific CTL, resulting in the destruction of TAA-expressing tumor cells (Dees et al., 2004; Svane et al., 2004). However, a major disadvantage of their use is that the production procedures are difficult, expensive and time-consuming. Indeed, the DC need to be isolated from the cancer patient, expanded in vitro, then loaded with peptide and then re-injected into the patient, all under sterile conditions. It is difficult to obtain sufficient viable DC with this approach. To circumvent these difficulties, several clinical trials in patients with melanomas, and breast or prostate cancer, have been performed with some success using TAA-peptides without DC but in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF; Markovic et al., 2006; Peoples et al., 2005, Perambakam et al., 2006). A disadvantage of these latter clinical trials is the high (toxic) concentrations of TAA-peptides required to obtain sufficient DC with MHC/TAA-peptide complexes in vivo. It is difficult to load DC with peptides in vivo, because the injected peptides need to compete with existing peptides associated with the MHC molecules at the membrane of DC. In addition, the number of epitopes presented by peptide-based vaccines are limited compared to tumor-cell-dendritic hybrid- or DNA based vaccines, as discussed below.

2.2 Tumor Cell-Dendritic Cell Hybrid Vaccines

Generation of hybrids between allogeneic tumor cells and autologous DC, presenting antigens expressed by the tumor in concert with costimulating capacities of DC is another approach to activate TAA-specific CTL (Gong et al., 2000). An

advantage is that a broad variety known and unknown TAA are included in this type of vaccine, and the TAA are now expressed in the presence of high levels of self-MHC and co-stimulatory molecules. Also allogeneic DC has been fused with autologous tumor cells resulting in the activation of TAA-specific CTL (Kugler et al., 2000). Caution must be taken to avoid activating autoimmunity against normal cells (Grossman and Paul, 2000; Nair et al., 2000). Human clinical vaccine trials with tumor cell-dendritic cell hybrid vaccines showed promising results in metastatic breast and renal cancer, but the procedures are as difficult as with peptide-based vaccines.

2.3 DNA-Based Vaccines

Use of DNA vaccines allows activation of TAA-specific CTL. Like the above described DC-based vaccines, DNA-based vaccines circumvent the poor APC function of the tumor cells since the antigens delivered by the DNA vaccines will be presented by professional APC that do express high levels of MHC and costimulatory molecules. A conventional DNA vaccine is a bacterial plasmid (for instance pCDNA3.1) containing an eukaryotic promoter (required for transcription), a Kozak sequence (required for translation) and the gene of interest, followed by a polyadenylation signal (to prevent degradation of mRNA). The gene of interest can be any DNA sequence that may activate tumor-specific T-cell responses.

Intramuscular or epidermal immunization with a DNA vaccine leads to DNA uptake into APC such as bone marrow-derived DC, macrophages, or Langerhans cells (Dupuis et al., 2000). CpG-rich motifs (high frequency of unmethylated CG sequences) present in bacterial DNA, binds to APC, internalizes via a clathrin-dependent endocytic pathway, and then rapidly moves into a tubular lysosomal compartment, where it binds to Toll-like receptor (TLR-9), initiating signal transduction (Latz et al., 2004), followed by activation and maturation of APC (Jakob et al., 1999). Cutaneous bombardment with DNA, using the gene gun is different from epidermal or intramuscular immunization (for a review see Boyle and Robinson, 2000). It results in direct delivery (physically) of the DNA into the cytoplasm of Langerhans cells. These DC migrate to regional lymph nodes in order to present antigens delivered by the DNA vaccine to naïve CTL. Another promising candidate vaccine vector is *Listeria monocytogenes (L. monocytogenes)*, because it naturally infects professional APC (monocytes), and targets antigen delivery to both the class I MHC pathway of endogenous antigen presentation and the class II MHC pathway of exogenous antigen presentation. This DNA delivery system (containing the same antigen as expressed by the tumors) successfully protected mice from renal or colorectal tumors (Pan et al., 1995). Advantages of *L. monocytogenes* are the higher efficiency of DNA uptake into APC and subsequent processing and antigen presentation compared to the conventional DNA immunizations described above, and the possibility of oral administration. Currently, most research in DNA vaccines is focused on the improvement of DNA uptake and target specificity.

Of all three types of cancer vaccines discussed in this section, DNA-based vaccines are the most promising. DC-based vaccines are difficult to prepare and expensive. In contrast, once developed, DNA-based vaccines are much more attractive in this respect, i.e., higher stability, lower costs. The most important advantage of DNA-based vaccines is the numerous possibilities to eliminate T-cell unresponsiveness. DNA-based vaccines allow inclusion of multiple genes into the DNA vector that may lead to enhanced T-cell responses, simultaneously with the TAA-encoding gene(s). Examples are genes for IL-2, IFNγ, heat-shock proteins HSP-70, or adjuvants (Chen et al., 2000; Lusgarten et al., 1999).

Before reviewing vaccination in preclinical and clinical trials, I will first discuss the single and most important hurdle to successful vaccination against cancer, i.e., reduced T-cell unresponsiveness in cancer patient and at older age.

3 Diminished T-cell Responses in Cancer Patients

CTL are considered to be the most important players in antitumor reactions. The TCR of CTL recognizes TAA in association with MHC molecules on the tumor cells. As has become evident from in vitro studies, these CTL are activated when exposed simultaneously to both TAA/self-MHC complexes and costimulatory molecules, resulting in CTL-mediated tumor cell cytolysis. In cancer patients, CTL are often found at the site of the tumor, but have evidently been unable to destroy the tumor cells (Gravekamp et al., 1990).

Multiple possible causes have been described for this unresponsiveness of the CTL in cancer patients (for a review, see Gravekamp 2001; Schreiber, 1998). For example, low expression of the costimulatory molecule B7.1 on human metastatic carcinoma cells in gastrointestinal tumors (Koyama et al., 1998) has been suggested as a possible cause of T-cell unresponsiveness because the interaction between costimulatory molecules and its ligand is an absolute need for T-cell activation. Other described costimulatory or adhesion molecules that may play a role in T-cell unresponsiveness are intercellular adhesion molecules (ICAM)-1, vascular cellular adhesion molecules (VCAM)-1 or epidermal lymphocyte adhesion molecules (ELAM)-1 (Maurer et al., 1998). In mice, the importance of co-stimulatory molecules such as 4-1BBL or B7.1, for CTL activation, has been reported as well (Loo et al., 1997; Melero et al., 1997).

T-cell unresponsiveness could also be due to low expression of self-MHC on tumor cells, which is required for recognition of tumor-specific antigens by the TCR. Low expression of MHC molecules has been commonly found in human metastatic tumor cells, such as metastatic melanoma, breast cancer or colon cancer (Garrido et al., 1997). Similarly, low expression of MHC molecules has been observed in mouse tumors, such as primary brain tumors (Akbasak et al., 1991), fibrosarcoma (Pedrinaci et al., 1999), melanoma (Weber and Rosenburg, 1990), or lung carcinoma (Blieden et al., 1991). As discussed later in this article, vaccination based on TAA could circumvent both problems. Even then, however, loss of TAA

expression will allow tumor cells to escape vaccine-induced T-cell responses (Corver et al., 2000; Sypniewska et al., 2005). This underlines the need of multi-antigen vaccines.

T-cell tolerance may be the most important obstacle for successful immunotherapy against cancer (for a review, see Zou, 2006). Since most TAA are weakly expressed on normal cells, the immune system will recognize them as self. Earlier in life T-cells are taught in the primary lymphoid organs not to respond to self-antigens. Therefore, it is difficult to induce a strong immune response against most TAA. Evidence exists in humans and mice that natural regulatory T-cells (T_{regs}), expressing $CD4^+CD25^+$, are crucial for maintaining T-cell tolerance to self-antigens. More recent studies have shown that transcription factor forkhead box P3 (FOXP3) plays a critical role in the development of functional T_{regs} ($CD4^+CD25^+FOXP3^+$). Natural T_{regs} are different from inducible T_{regs}. Inducible T_{regs} arise during inflammatory processes such as infections and cancers (for a review see Curiel, 2007). Although inducible T_{regs} express CD4, CD25, and FoxP3 like natural T_{regs}, they are functionally different. In contrast to natural T_{regs}, inducible T_{regs} suppress immunity through the production of soluble factors such as IL-10 and transforming growth factor (TGF)β or through direct cell-cell contact (Bluestone and Abbas, 2003). It has been shown in mice (Shimizu et al., 1999; Tanaka et al, 2002) and man (Chen et al., 2007, Manhke et al., 2007) that inducible T_{regs} inhibits vaccine-induced T-cell responses as well as NK cell responses. Many strategies have been discussed and tested in animal models that target the inactivation or elimination of inducible T_{regs}, in order to enhance vaccine efficacy. For instance, treatment with a tumor cell-based vaccine secreting granulocyte-macrophage colony stimulating factor (GM-CSF), combined with anti-CTLA-4 antibodies (CTLA-4 is constitutively expressed in T_{regs}) in the B16/B6 melanoma mouse tumor model resulted in eradication of primary tumors and prevention of lung metastases. This was accompanied with improved anti-tumor responses (van Elsas et al., 1999). However, in humans with stage IV melanoma or renal cancer administration of anti-CTLA-4-specific antibodies did not inhibit suppressive activity of T_{regs} in vitro or in vivo (Maker et al., 2005). Recently, it has been reported that also $CD4^+CD25^+FOXP3^-$ are suppressive and that not all $CD4^+CD25^+FOXP3^+$ are suppressive (Gavin et al., 2007; Wan et al., 2007). Inducible T_{regs} may become an important target for cancer immunotherapy, but more research is needed to identify which $CD4^+CD25^+$ T-cells are suppressive, and how inducible T_{regs} can be targeted without depletion of natural Tregs. Depletion of natural T regs leads to autoimmune diseases (Sakaguchi et al., 1995).

Yet, other factors may play a role in inhibition of vaccine-induced T-cell responses in cancer patients. In humans and mice, many tumors secrete lymphokines or factors that inhibit vaccine-induced immune responses. Examples are TGFβ, IL-6, IL-10, cyclooxygenase-2 (COX-2) and its products prostaglandine E2 (PGE_2), PD1-ligand, or indolamine 2,3-dioxygenase (IDO; Gajewski et al., 2006). TGFβ inhibits antigen presentation by DC, resulting in inhibition of T-cell function (Kobie et al., 2003), or facilitates the induction of T_{regs} resulting in suppression of tumor-specific CD8 T-cell cytotoxicity in vivo (Chen et al., 2005). IL-6 is a potent regulator of DC differentiation in vivo (Park et al., 2004), and is able to initiate the expression of

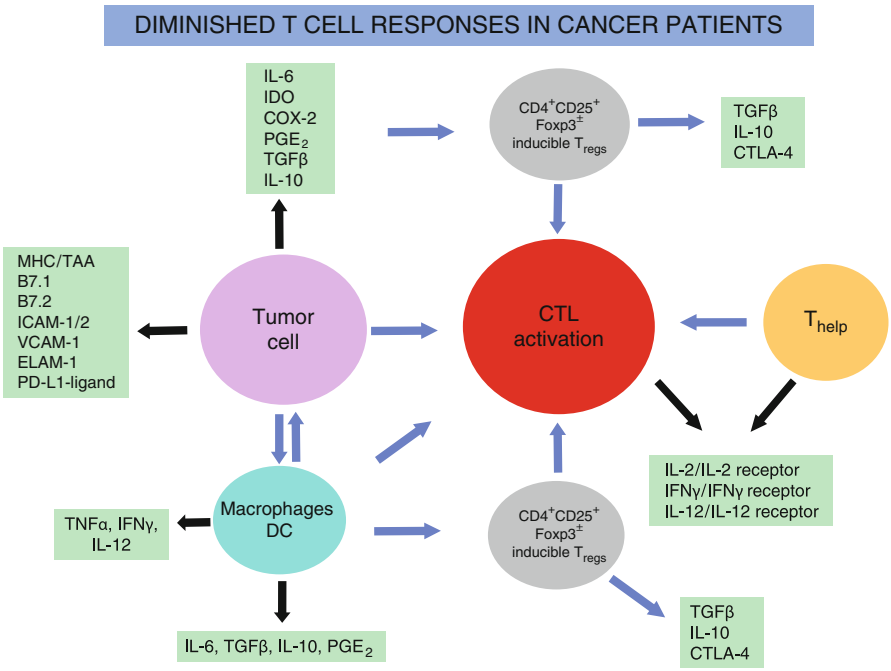

Fig. 1 Diminished T-cell responses in cancer patients and in mouse tumor models. MHC/TAA, B7.1/2, ICAM-1/2, VCAM-1, ELAM-1, and PD-1L-ligand are expressed on the membrane of tumors cells

These molecules are often weakly expressed or altered or even absent in cancer patients resulting in diminished CTL responses. IL-6, IDO, COX-2, PGE_2, TGFβ, and IL-10 are lymphokines or factors produced by tumor cells, inhibiting vaccine- and tumor-induced CTL cell responses. TGFβ, COX-2 and its product PGE_2 facilitate the generation of inducible $CD4^+CD25^+FOXP3\pm$ regulatory T cells (T_{regs}). Inducible T_{regs} can be $FOXP3^+$ or $FOXP3^-$, and inhibits the production of IFNγ and IL-2 secreted by CTL and/or T_{help} cells, through the production of TGFβ and IL-10 or through cell-cell contact. Inducible T_{regs} express CTLA-4 constitutively. CTLA-4, the alternative ligand for B7.1 is an inhibitory receptor limiting T cell activation. PGE_2 can also down regulate IL-12, a lymphokine important for long-term CTL responses after interaction with the IL-12 receptor. IL-6 may prevent maturation of APC, and subsequent antigen presentation and T-cell activation. IDO may inhibit T cell activation through tryptophan degradation. IL-10 inhibits maturation of DC and T-cell function through T_{regs}. Many of these factors such as IL-6, TGFβ, IL-10 and PGE_2 can also be produced by macrophages and/or dendritic cells, resulting in inhibition of CTL responses. However, there are also macrophages that can activate CTL by the production of IFNγ or IL-12, or induce Fas-mediated apoptosis of tumor cells by CTL through the production of TNFα, or kill tumor cells directly by the production of TNFα and IFNγ

APC, antigen-presenting cells; COX-2, cyclooxygenase-2; CTL, cytotoxic T lymphocytes; DC, dendritic cells; ELAM, epidermal lymphocyte adhesion molecule; FoxP3, forkhead box P3; ICAM, inter cellular adhesion molecule; IDO, indolamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; MHC, major-histocompatibility complex; PGE, prostaglandine E; TAA, tumor-associated antigens; TGF, transforming growth factor; Thelp, helper T cells; Tregs, regulatory T cells; VCAM, vascular cellular adhesion molecule.

signal transducer and activator of transcription (STAT)3 in DC. However, high levels of STAT3 can prevent the maturation from DC, and subsequent presentation of antigens (Park et al., 2004), resulting in T-cell inhibition. IL-10 inhibits maturation of DC and T-cell function through T_{regs} (Jonuleit et al., 2001). COX-2 and its product PGE_2 induce the expression of T_{reg} cell-specific transcription factor Foxp3 and increase T_{reg} activity (Sharma et al., 2005). PGE_2 also downregulates IL-12 production by macrophages and differentiation of Th1 responses (Kuroda and Yamashita (2003). Engagement of PD-1 by PD-1 ligand leads to inhibition of T-cell receptor-mediated lymphocyte proliferation and cytokine secretion (Freeman et al., 2000). IDO inhibits T-cell proliferation through tryptophan degradation (Hwu et al., 2000). Reduction of these lymphokines or factors may enhance vaccine efficacy.

Over the last few years, more attention has been given to macrophages. Macrophages in the microenvironment of tumors, may also produce IL-6, TGFβ, PGE_2, and IL-10, and subsequently inhibit T-cell activation (Kim et al., 2006). However, macrophages may also stimulate T-cells through the production of IFNγ or IL-12 (Sica et al., 2000), or produce TNFα facilitating Fas-mediated tumor cell apoptosis by CD8 T-cells (Starace et al., 2004), or become so-called "killer macrophages" when producing TNFα and IFNγ (Baron-Bodo et al., 2005; Ouyang et al., 2006). Macrophages play a crucial role in suppression and activation of the immune system in cancer patients, and may become an important target for anticancer therapies.

A schematic overview of impaired T-cell responses in cancer patients is shown in Fig. 1. To develop effective cancer vaccines, many of the above-described obstacles have to be overcome. However, an additional problem almost totally ignored in the development of cancer vaccines, is ageing of the immune system.

4 Diminished T-cell Responses in Elderly

Ageing of the immune system leads to impaired T-cell responses in elderly, including noncancer patients (Miller et al., 1996). Below, alteration in several immunological parameters that may contribute to the age-related decline in T-cell responses is discussed. In this discussion, human and mouse are compared. For instance, a decrease in the number of naive T-cells (capable of reacting to new antigens) and an increase in the number of memory T-cells (capable of reacting to previously exposed antigens) in elderly humans as compared to young adults have been reported (Utsuyama et al., 1992). It has been suggested that continual activation of the immune system by new antigens during the life span would lead to a depletion of naive T-cells from the thymus, and a clonal expansion of memory T-cells. With the involution of the thymus almost complete at the age of 60 years, new naive T-cells at old age can no longer be generated (Grubeck-Loebenstein, 1997). The host is then dependent on the pool of naïve T-cells generated earlier in life. Analogous to the situation in humans, a decrease of naïve T-cells and an increase of memory T-cells have also been described for aging mice (for a review see George and Ritter, 1996). Other possible causes for diminished T-cell responses in aged humans and mice have been described, such as

defects in TCR/CD3-mediated phosphorylation events or aberrant regulation of tyrosine kinases associated with the TCR (Tamir et al., 2000). An age-related decrease in the αβ repertoire of the human TCR has been described that may lead to diminished T-cell responses (Wack et al., 1998). Another molecule important for T-cell activation is CD28. CD28 is expressed at the cell membrane of T-cells, and is the ligand for the co-stimulatory molecule B7, expressed on APC. Clinical studies have documented that high proportions of CD8 T-cells that lack CD28 are correlated with a reduced antibody response to influenza vaccination (for a review, see Effros, 2006). Also in mice, CD8 T-cells lacking CD28 expression have been reported (for a review, see Effros, 2004). Moreover, it has been shown that CD28-lacking CD8 T-cells can suppress antigen-specific CTL responses (Filaci et al., 2004).

Studies have been performed in the mouse to evaluate the involvement of natural T_{regs} in age-related decline in T-cell-mediated immune responses. Suggestive evidence exists that age-related decline in immune responses is ascribed to changes in the $CD4^+CD25^-Fox3^+$ T-cell population and not to a functional augmentation of suppressive $CD4^+CD25^+Fox3^+$ T_{regs} (Nishioka et al., 2006). Others have shown that the number of human peripheral blood CD4+$CD25^{high}$ T_{regs} increased with age but their function appeared to be unaltered in comparison to young age (Gregg et al., 2005). The relevance of CD4+$CD25^{high}$ T_{regs} in relation to immune senescence as yet remains unclear.

In addition to the problems at the level of T-cells and/or tumor cells, defects in cytokine production have been observed in aged humans. An example is a human vaccine study in which significantly lower IL-2 was produced by T-cells of older individuals stimulated with an influenza vaccine in vitro compared to those of young individuals (McElhaney et al., 1994). Similarly, significantly lower IFNγ was produced by peripheral blood mononuclear cells (PBMC) from elderly individuals immunized with an influenza vaccine compared to young individuals (Quyang et al., 2000). IL-2 promotes T-cell activation and proliferation, as well as release of IFNγ by T-cells. The lower IL-2 production following in vitro stimulation with the influenza vaccine may explain the lower IFNγ production. IFNγ is involved in activation of APC such as macrophages. These macrophages are important for CTL priming.

Defects in other cell types than CTL and/or tumor cells may also explain T-cell unresponsiveness in aging. Antigen presenting cells (APC), such as DC in blood or Langerhans cells in skin, play a central role in T-cell activation. These cells are often called "professional" APC because they enable efficient processing of foreign proteins into peptides, and because they express MHC and costimulatory molecules at the high levels required for optimal presentation of the peptides to the immune system and subsequent stimulation of T-cells. Tumor cells are poor APC, because costimulatory molecules and self-MHC, as well as TAA required for CTL stimulation are often weakly expressed. Therefore, DC loaded with peptides or fused with autologous tumor cells are frequently used in clinical trials as an anticancer immunotherapy to stimulate T-cells in cancer patients. Since APC play such an important role in T-cell activation, one might question whether this cell type could be involved in the age-related decline of T-cell responsiveness. Different results have been published about the function of DC at older age. For instance, it has been

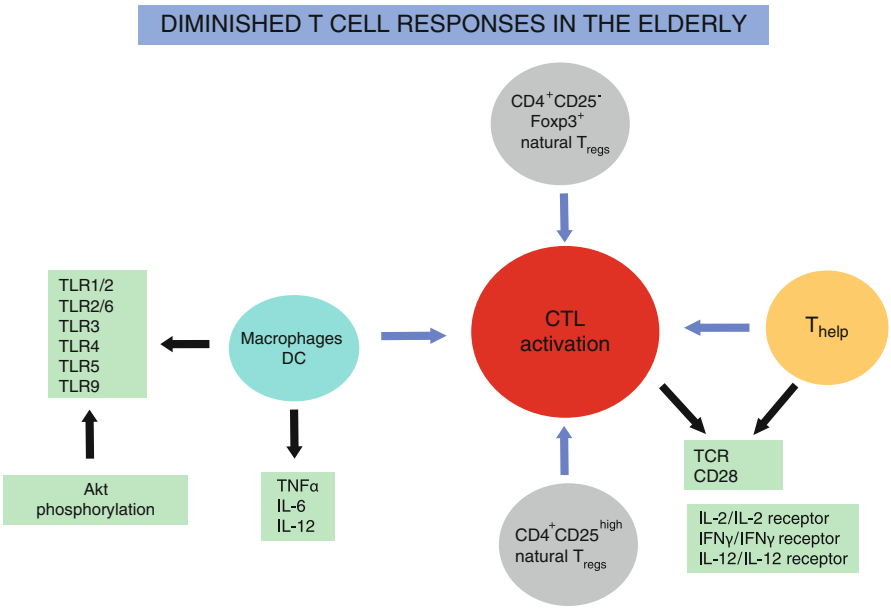

Fig. 2 Diminished T cell responses in elderly and old mice. Ageing of the immune system leads to impaired T cell responses in elderly and old mice. Decrease in the number of naïve T cells (capable of reacting to new antigens) during life is probably the most observed age-related phenomenon in human and mice

However, many ligands and receptors on T cells and APC, and lymphokines produced by T cells and APC play an important role in age-related diminished T cell responses as well. Those discussed in this review are shown here. The TCR is expressed by T cells, and recognizes antigens in association with self-MHC. An age-related decrease in the αβ repertoire of the TCR and defects in TCR/CD3-mediated phosphorylation events has been observed at older age. Lack of CD28, a ligand for co-stimulatory molecules B7.1/2, expression on T cells is associated with diminished T cell responses at older age. Decreased production of IL-2 and/or IFNγ, both required for T cell stimulation, has been observed at older age in mice and humans. It has been shown in the elderly that natural $CD4^+CD25^-FoxP3$ but not $CD4^+CD25^+$ FoxP3 T_{regs} contributes to T cell inhibition. In another study it has been shown that $CD4^+CD25^{high}T_{regs}$ increases with age but are functional similar to natural $CD4^+CD25^{high}T_{regs}$ of young individuals. It has been shown that IL-12 is less produced by DC of aged compared to young individuals. Phosphorylation of AKT in DC of aged individuals, indirectly leads to decreased phagocytosis and migration, and regulates TLR signaling in DC and macrophages. TLR signaling activates the innate and adaptive immune system. In contrast to DC, macrophages secrete lower levels of IL-6 and TNFα when stimulated with ligands for the TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR9 in young compared to old mice. Other ligands, receptors and/or lymphokines than presented in this figure may play a role in age-related decline of T cell responses as well but have been not reported so far.CD, cluster differentiation; CTL, cytotoxic T lymphocyte; DC, dendritic cells; FoxP3, forkhead box P3; IL, interleukin; IFN, interferon; Thelp, helper T cells; TCR, T cell receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor; Tregs, regulatory T cells.

demonstrated that blood DC from old individuals can still function as powerful APC when exposed to purified protein derivate (PPD) of *Mycobacterium tuberculosis* (Lung et al., 2000). Similarly, the responsiveness of blood DC to stimulation with influenza vaccine was unimpaired at old age (Sauerwein-Teissl et al., 1998). In both cases, expression patterns of MHC molecules and costimulatory molecules were found to be similar on blood DC of young and old individuals. However, it has been shown in mice and humans that the number of Langerhans cells decreases with age, resulting in impaired immune function of the skin (Sprecher et al., 1990; Sunderkotter et al., 1997). It has also been shown that DCs from aged individuals are more mature and have impaired ability to produce IL-12 (Bella et al., 2007), or that secretion of TNFα and IL-6 significantly increased upon stimulation with LPS and ssRNA in DC of aged compared to young individuals (Agrawal et al., 2007). Others found reduced phosphorylation of Akt in DCs of aged individual (Agrawal et al., 2007), resulting in decreased activation of phosphatidylinositol 3-kinase (PI3K) pathway. P13K-signaling regulates phagocytosis, and migration, as well as TLR signaling. TLR are pattern recognition receptors that recognize conserved molecular patterns on microbes and activate the innate and adaptive immune system. In contrast to DC, macrophages appeared to secrete significantly lower levels of IL-6 and TNFα when stimulated with known ligands for the TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR9 in old than young mice (Renshaw et al., 2002).

Hence, in cancer patients, T-cell functions are not only inhibited by the tumors but also impaired as a result of aging. Figure 2 depicts ligands and receptors on T-cells and/or APC, as well as factors secreted by T-cells and/or APC involved in diminished T-cell responsiveness at old age.

5 Preclinical Mouse Tumor Models

The mouse is undoubtedly the most suitable preclinical model for testing the potential efficacy of cancer vaccines in humans as a function of age. Mice are evolutionary close to human and can be economically maintained. Aging-related immune responses in the mouse are very similar to that of humans although distinct differences are also present, such as the organization of MHC, TCR, or immune globulin structures (or a review see Davis and Chien, 1998; Margulies, 1998; Max, 1998). However, there is no organism apart from humans of which so much is known immunologically. Mice are also extremely well defined genetically and like humans their genome is completely sequenced. Indeed, at least ten different mouse lab strains and four wild mice have been totally sequenced (Callaway, 2007). As much as 99% of all mouse genes, have a human counterpart. Finally, the mouse is now also increasingly well characterized phenotypically with computerized databases of all forms of aging-related pathology readily available (Calder et al., 2007).

Here, I will discuss various types of preclinical mouse tumor models and their usefulness for predicting age effects on cancer vaccination. Suitable preclinical

models, that adequately reflect human cancer, may teach us how to overcome the problems that occur in cancer patients in order to develop effective cancer vaccines. Criteria for suitable models are immune competence, developing human-like tumors, and expressing self-TAA.

Most studies report about the use of syngeneic models. In this type of models, a syngeneic tumor cell line (same genetic background as the receiving mouse) is injected into the mouse and tumor formation is within 1–4 weeks. Advantages of these models are the fast results obtained and the possibility to test vaccines at young and old age. An overview of available syngeneic mouse tumor models can be found in Current Protocols in Immunology (Ostrand-Rosenberg and Kruisbeek, 2000). However, transgenic models reflect human cancer more adequately, since the development of cancer in these models is more natural, i.e., they undergo normal-preneoplastic-neoplastic stages. These models are more useful to develop preventive vaccines. The Mouse Models of Human Cancers Consortium provides an overview of available transgenic mouse tumor models at website http://mouse.ncifcrf.gov. Disadvantages are the time frame in which the vaccines can be tested, since it takes 3–7 months before tumors appear in transgenic mice, which makes it expensive, and vaccines can only be tested at a relatively young age. Therefore, transgenic mice are not useful for developing vaccines at older age. Inducible conditional mouse tumor models, in which tumor development can be induced at young and old age by the administration of antibiotics or drugs, are most suitable (for a review see Jonkers and Berns, 2002). Although most of these models have been tested for tumor development at young age only, they exhibit the potential to develop tumors at old age when the antibiotic or drug is administered at old age. The disadvantage of these models is the time frame necessary to induce the tumor (3–7 months), which makes it expensive, and technical problems that may occur such as leakage (overexpression of tumor-inducing gene without administration of antibiotic or drug). An overview of inducible conditional mouse tumor models that could be useful to develop cancer vaccines at older age is given in Table 1.

Table 1 Inducible conditional mouse tumor models

Cancer	Model	Inducing agent	References
Lung	Lp-stopLp-K-ras G12D	Ad-Cre	Jackson et al. 2001
Cervical cancer	K14-HPV16	Estrogen	Elson et al. 2000
Sarcoma/Epithelial tumors/ Lymphoma	p53S389A	Spontaneous/UV	Bruins et al. 2004
Melanoma	TyrP-LpCreERLp-iRasIRES-P1A/Lp-Ink4A/ARF-Lp	Tamoxifen	Huijbers et al. 2007
Liver tumors	AlbP-Lp-stopLp-Luc-2A-GFP/AlbP-Lp-stopLp-SV40-tag	Ad-Cre	Hammerling, 2007

Ad = Adenovirus, AlbP = Albumin promoter, Cre = cre-recombinase, GFP = green fluorescent protein, HPV = human papilloma virus, IRES = internal ribosome entry site, Lp = Lox P, Luc = luciferase, SV = simian virus, TyrP = Tyrosinase promoter.

6 Comparison of Cancer Vaccination in Preclinical Models and Human Clinical Trials

Many studies in preclinical models have become available in the last few years and results encourage vaccination as a nontoxic effective therapy against cancer. Table 2 summarizes the most effective and recent preclinical studies using syngeneic and transgenic mouse models. With respect to tumor protection, DNA-based Her2/neu vaccines were most effective in transgenic breast tumor model neuT (Quaglino et al., 2004, Spadaro et al., 2005). Complete protection was obtained up to 1-year. However, to decrease morbidity and mortality prevention or elimination of metastases is crucial. In this respect, Mage-b DNA, GRP94/T41, and mOX40L/GM-CSF, proved most effective in metastatic breast tumor models 4TO7cg and 4T1 (Ali et al., 2004; Liu et al., 2005; Sypniewska et al., 2005).

The results of these preclinical studies are promising. However, human clinical trials do not reflect these promising results. Table 3 summarizes the results of most recent and promising human clinical trials of various types of cancer treated with vaccines. The most successful vaccine is human papilloma virus (HPV), capable of complete prevention of cervix carcinoma, up to 3.5 years (average; Mao et al., 2006). A main reason for this success might be that the HPV-16 vaccine contains a viral antigen and will be recognized as foreign. Important is that HPV-16 vaccination is preventively effective. The potential negative effects of tumors on vaccine-induced immune responses that may occur in therapeutic vaccinations can be circumvented in preventive vaccinations. Like in the preclinical models, vaccination with MAGE (against metastatic melanoma) again showed regression of metastases (Salcedo et al., 2006). Also vaccination with TAA of survivin showed regression of metastases (Wobser et al., 2006). Although HER2/neu vaccination was promising, the efficacy was lower than in the preclinical models, i.e., some reduction in recurrence and prolonged stable disease (Dees et al., 2004; Peoples et al., 2005). Overall, the results from preclinical models were more successful than results from human clinical trials. One reason might be that most vaccines in the preclinical models have been tested preventively, and therapeutic vaccinations in the preclinical models may have started relatively earlier when tumors were very small or not palpable yet, compared to vaccinations in human clinical trials. However, another or additional important reason might be the age of cancer patients at the time of vaccination, who were generally over 50 years of age. As shown below, the age is an important factor for efficacy of cancer vaccines.

7 Comparison of Cancer Vaccination at Young and Old Age in Preclinical Models

More than 50% of all cancer patients are 65 years or older (Muss, 2001). However, as discussed earlier, the immune system at older age is impaired, due to T-cell unresponsiveness. In almost all preclinical studies, cancer vaccines have been tested at

Table 2 Cancer Vaccination in Preclinical Models

Cancer	Antigen	Vaccine	Results So Far	Animal model	References
Melanoma	Tumor lysate	Fls/DC	RTG	B16/syng/P	Yoshikawa et al., 2006
	Tumor lysate/CpG	DC/DNA	RTG/PS	B16/syng/P/T	Pilon-Thomas et a., 2006
Breast	Her2/neu/extrcell.	DNA/electroporation	CP up to 1 year $p<0.05$	neuT/transg/P/T	Quaglino et al., 2004
	Her2/neuIL-12	DNA electroporation	CP up to 1 year $p<0.05$	neuT/transg/P.	Spadaro et al., 2005
	Her2/neu	DNA	CP up to 60 days $p<0.05$	Her2/neu/syng/P	Chan et al., 2006
	Her2/neu	DC/neu	TD $p<0.05$	Her2/neu/transg/P	Chan et al., 2006
	Her2/neu	Listeria	TR/Her2/CTLR $p<0.05$	Her2/neu/syng/T	Singh et al., 2005
	Mage-b	DNA	PM $p<0.05$/RTG $p<0.05$	4TO7cg/syng/P	Sypniewska et al., 2005
	BORIS/CD80	DNA/Adjuvant	PS/RTG	4T1/syng/P	Loukinov et al., 2006
	GRP94/4T1	DNA/Radiation	PM/TD $p<0.05$	4T1/syng/T	Liu et al., 2005
	mOX40L/GM-CSF	Fusion protein	PM/RTG $p<0.05$	4T1/syng/P/T	Ali et al., 2004
Ovarian	IL-2/TGFb-AS	Fibroblast/Tumor	TP $p<0.01$	MOT/syng/P	Dorigo et al., 1998
Colon	mOX40L/GM-CSF	Fusion protein	RTG	CT26/syng/P/T	Ali et al., 2004
	SVF10-E-IL18	RNAsuicide vector	TR	CT26/syng/T	Chikkanna et al., 2006
	AFP	S.typhimurium	ILS $p<0.05$	CT26/syng/P	Chou et al., 2006
Prostate	CDL40	Adenovirus	RTG $p<0.001$	TRAMP-C2/syng/P/T	Dzojic et al., 2006
	Tumor/AdIL-12	Adenovirus	TF 40% $p<0.01$	TRAMP-C2/syng/T	Nikitina et al., 2005
	AdIL-12+Adicasp1	Adenovirus	ILS 62% $P=0.03$	TRAMP-C2/transg/T	Nikitina et al., 2005
AML	AML peptides	DC/FTA peptides	ILS $p<0.0001$	C1498/syng/P	Delluc et al., 2007

AFP=alpha feto protein, AML= acute myeloid leukemia, CP=complete protection, CTLR=cytotoxic T lymphocyte responses, DC= dendritic cells, FLs=fusogenic liposomes, FTA=trifluoroacetic acid, GM-CSF=granulocyte macrophage-colony stimulating factor, GRP=glucose-regulated protein, HCC= hepato cellular carcinoma, IL=interleukin, ILS=increased life span, Mage=melanoma-associated antigen, MOT= murine ovarian teratoma, P=preventive vaccination, PM=prevention metastases, PS=prolonged survival, RTG=reduced tumor growth, SFV-10E= enhanced Semliki Forest virus, T=therapeutic vaccination, TF=tumor free, TD=tumor delay, TGF=transforming growth factor, TP=tumor prevention, TR = tumor regression.

young age (Table 3). Very recently, results of a few vaccine studies in preclinical models at old age became available, including studies with Mage-b vaccination performed in our laboratory. These are the first studies that show that cancer vaccines are less effective at old than at young age.

The first reported vaccine study in young and old mice with cancer is from Provinciali et al. in (2000). A syngeneic mammary adenocarcinoma cell line TS/A was genetically engineered to release IL-2 (TS/A-IL-2). Young and old mice were immunized with TS/A-IL-2 cells and subsequently challenged with the parental TS/A cell line. While TS/A-IL-2 protected 90% of the young mice, only 10% was protected of the old mice. CD8 and CD4 T-cells were detected in tumors of young but hardly in tumors of old mice, while macrophages and neutrophils were abundantly present in tumors of mice from both ages. Yet, it has not been proven whether these CD8 and CD4 T-cells were functionally active.

Many vaccine studies have been performed with the Her2/neu DNA vaccine in young mice. Provinciali developed a pCMVneuNT DNA vaccine and tested its efficacy in young and old mice that were subsequently challenged with syngeneic TUBO cells, overexpressing HER2/neu (Proviciali et al., 2003). Young mice were completely protected while less than 60% of the old mice were protected against TUBO challenge. Anti-neu antibodies, induced by the vaccine, and proliferation after restimulation in vitro, was higher at young than at old age.

Lusgarten et al., (2004) immunized young and old mice with a syngeneic pre-B cell lymphoma cell line (BM-185), expressing enhanced Green Fluorescent Protein (EGFP) and a costimulatory molecule CD80 (B7.1). While the young mice developed a long-lasting memory response capable of rejecting BM-185 wild type tumors, the old mice did not develop long-lasting memory responses. However, when the BM-185-EGFP-CD80 plus agonist anti-OX40 mAb were injected in old mice, long-lasting memory responses were capable of rejecting BM-185 wild type tumor cells with the same vigor as in young mice. In vivo depletion of CD8 T-cells resulted in decreased survival of mice immunized with BM-185-EGFP, and challenged with wild type BM-185. However, these studies were performed at young age only. They also analyzed the number of CD8 T-cells specific for EGFP and found cytotoxic activity against BM-185 wild type tumor cells in spleen cultures stimulated with BM-185-EGFP-CD80 in old and young mice. However, the spleen cultures were from mice without tumors. In another study by the group of Lusgarten, DC vaccination plus rIL-2 protected 60% of the young mice from challenge with syngeneic TRAMP-C2 tumor cells (adenocarcinoma of the prostate), while only a minimal effect was observed in the old mice (Sharma et al., 2006). However, when coadministered with anti-OX-40 or anti-4-1BB mAbs (leukocyte differentiation antigen on T and NK cells, and DC) a vigorous anti-tumor response in both young (85–90%) and in old (70–75%) mice was observed. Cytolytic activity in spleen cultures of young and old mice immunized with DC pulsed with apoptotic TRAMP-C2 cells, and anti-OX40 or anti-4-1BB antibodies were observed. However, the spleen cultures were from mice without tumors.

Table 3 Cancer vaccination in clinical trials

Cancer	Antigen	Vaccine	Results So Far	References
Melanoma	MAGE-A1/3	Tumor DC/Tumor lysate	TR	Salcedo et al., 2005
	MART-1/gp100/tyros	Peptide/low dose GM-CSF	ICO	Markovic et al., 2006
	VACCIMEL	Allogeneic melanoma/BCG	HR $p<0.05$	Barrio et al., 2006
	IL-2/Difteria-toxin fusion/	Protein/peptides	Treg reduced,	Mahnke et al., 2007
	MelanA/MART-1/gp100		spec CD8 increased $p<0.01$	
Breast	1E10 (GM3 ganglioside)	anti-Id Ab	HR/CR $p<0.05$	Guthmann et al., 2006
	Membrane Ags	DC/Tumor	SD/DR/TCR	Avigan et al., 2004
	HER2/neu	HER2/neupeptide/DC	SD/TCR	Avigan et al., 2004
	HER2/neuE75	Peptide/GM-CSF	RR $p<0.19$/PSIR	Peoples et al., 2005
	P53	Peptide/DC	SD/TCR	Svane et al., 2004
Lung (NSCLC)	EGF/cyclophosphamide	protein/drug	spec HR, IS $p<0.05$	Gonzalez et al., 2007
Cervix Carcinoma	HPV-16L1	polypeptide/VLP	CP up to 48 months	Mao et al., 2006
Colon	B16/LCC	Tumor lysate/xenotypic	IS, CR, HR	Seledtsov et al., 2007
Pancreas	Survivin	Peptide/Adjuvant	RM/IR (case report)	Wobser et al., 2006
Renal Carcinoma	RNA Tumor	RNA transf DC/depl Tregs	TSTCR $p<0.05$	Dannull et al., 2005
Prostate	PSA	Peptide/GM-CSF	PSA-spec TCR	Perambakam et al., 2006
Follicular Lymphoma	Id epitope	Protein	DFS/HR/CR	Inoges et al., 2006

Ab=antibody, CP=complete prevention, BCG=Bacillus Calmette Guerin, CR=cellular responses, DC=dendritic cells, DFS=disease free survival, DR=disease regression, GM-CSF=granulocyte macrophage-colony stimulating factor, PSA=prostate specific antigen, HR=humoral responses, ICO=improved clinical outcome, Id=idiotypic, IR=immune responses, IS=increased survival, NSCLC=non small cell lung cancer, PSA=prostate specific antigen, PSIR=peptide specific immune responses, RM=regression metastases, RR=recurrence reduced, SD=stable disease, TCR=T cell responses, TR=tumor regression, Tregs=regulatory T cells, TSTCR=tumor-specific T cell responses, VLP=virus-like particle.

In our laboratory, we developed a DNA vaccine of Mage-b and tested this vaccine at young and old age in a syngeneic mouse tumor model, 4TO7cg. This mouse tumor model is moderately metastatic (range: 2–20 metastases per mouse) and overexpresses Mage-b in primary tumor and metastases (Sypniewska et al., 2005). Preventive vaccination of young and old mice with pcDNA3.1-Mage-b protected 90% of the young mice from metastases, while only 65% of the old mice remained free of metastases (Gravekamp, 2007). Analysis of spleen cells of tumor-bearing mice after in vitro restimulation, showed high levels of IL-2 and IFNγ at young age but undetectable levels at old age. We repeated this vaccine study in a much more aggressive metastatic model 4T1 (range: 5–300 metastases per mouse), also overexpressing Mage-b, but this time we mixed the pcDNA3.1-Mage-b DNA vaccine with plasmid DNA secreting GM-CSF. To recruit APC more effectively to the peritoneal cavity (pc), thioglycollate was injected into the pc, prior to each vaccination (Gravekamp, 2007). Thioglycollate-stimulated macrophages are not fully differentiated, highly express MHC class II, and are highly phagocytic (Cook et al., 2003). Evidence exists that thioglycollate-stimulated macrophages can function as APC (Rock et al., 1993). Although the effect in the young mice was stronger than in the old, a significant reduction in the frequency of metastases was observed in both young and old mice. However, when analyzing the draining lymph nodes of tumor-bearing mice, moderate levels of IL-2 and IFNγ were detected after restimulation at young age but not at old age. FACS analysis of the draining lymph nodes of Mage-b vaccinated tumor-bearing mice at young and old age after restimulation, showed CD4 and CD8 responses (intracellular IL-2 and/or IFNγ production) at young age but not at old age. At old age macrophages and NK cells were more active (intracellular production of IFNγ and IL-2 receptor expression), suggesting that the innate immune response may have contributed to the anti-tumor response in the mice.

Finally, an interesting article has been published very recently (Daftarian et al., 2007), which describes the therapeutic effect of a single immunization with HLA-A2-restricted HPV peptides (CTL epitopes and T_{help} epitopes), combined with CpG adjuvant and ISA51 adjuvant encapsulated in liposomes, on HPV-16-expressing syngeneic TCI/A2 tumors in aged transgenic HLA-A2 mice. Impressive was the fact that they were able to eradicate large tumors of 700 mm^3 after a single vaccination, and that the mice were protected from re-challenge with an E6/E7-expressing tumor cell line. However, the mice in this study were not really old (48–58 weeks old mice with a C57/BL6 background), and the results were not compared to young mice. They showed vaccine-induced CD8 T-cell responses in naïve mice without tumors, but not in mice with tumors. In addition, HPV peptides are foreign for mice, and induction of an immune response against foreign is not the same as developing immune responses against self-antigens. However, from clinical point of view this is an interesting study and reflects the human situation, since HPV is foreign to human, and patients with cervical cancer are relatively young, i.e., women over 50 years of are at very low risk (Saslow et al., 2002). An overview of preclinical vaccine studies in young and old mice with tumors is summarized in Table 4.

Table 4 Cancer vaccination in young and old mice

Cancer	Antigen	Vaccine	Animal model	Results so far		P/T	References
				Young	Old		
Breast	Tumor	Tumor/IL-2	TS/A/S	TP 90% p<0.05	TP 10%	P	Provenciali et al. 2000
	neuNT	DNA	TUBO/S	CTP 100% p<0.05	<60% PTP	P	Provinciali et al. 2003
	Mage-b	DNA	4TO7cg/S	MWOM 90%	MWOM 65%	P	Sypniewska et al. 2005
	Mage-b	DNA		p=0.0108	p=0.1939		
		GM-CSF	4T1/S	RM 67% p=0.0083	RM 88% p=0.0020	P	Gravekamp 2007
		Thioglycollate		RTG 69% p=0.0059	RTG 22% p=0.0757		
Prostate	DC	anti-OX-40 or anti-4-1BB Abs	TRAMPC2/S	RTG 85-90% p<0.05 LLMR	RM 67% p=0.0083 RTG 69% p=0.0059 LLMR	P	Sharma et al. 2006
B cell lymphoma	Tumor	CD80/Tumor Anti-OX40 Mab	BM185/S	CTP 100% p<0.05 CD8 involved	CTP 100% p<0.05 CD8 involvement NT	P	Sharma et al. 2006
Cervix carcinoma	E7	CTLepitopes Thelpepitopes Liposomes CpG adjuvant ISA51 adjuvant	HLA-A2/S	NT	TE 60% of mice	T	Daftarian et al. 2007

CTP=complete tumor protection, CTL=cytotoxic T lymphocyte, DC=dendritic cells, GM-CSF=granulocyte-macrophage colony stimulating factor, HLA=human leucocyte antigen, IL=interleukin, LLMR=long lasting memory responses, P=preventive, PTP=partial tumor protection, MWOM=mice without metastases, NT=not tested, TP=tumor protection, RS=reduction metastases, RTG=reduced tumor growth, S=syngenbeic, T=therapeutic, TE=tumor eradication.

8 Summary and Future Prospects

The main conclusion from studies in preclinical models at young and old age is that cancer vaccines are less effective at older than at younger age. These results may imply that vaccines may not be very effective in cancer patients, which are usually elderly, unless the vaccines are optimized for older age. The studies discussed in this chapter show the potential but also the need for tailoring cancer vaccination to old age. Below, a number of approaches are proposed that may contribute to further improvement of vaccine efficacy of cancer vaccination at older age.

Active immunization offers great promise for elderly cancer patients. A first immunization at young age, when sufficient naïve T-cells are still present, followed by boosting at old age may improve T-cell responses at older age. This approach has shown to be effective for improving Ab responses at older age in mice (Stacy et al., 2003). However, lack of naïve T-cells is not the only hurdle to overcome, and it is clear that the immune system needs help to activate T-cells against cancer cells. DNA vaccines are of great value since any DNA sequence can be added to the vaccine vector that may improve T-cell activation with minimal toxicity. Activation of T-cells at older age could be achieved by expressing IL-2 and IFNγ from the vaccine vector, or GM-CSG or Flt3-ligand to the vaccine vector in order to activate macrophages and DC or improve processing and presentation of antigens by APC. Addition of a DNA sequence encoding IL-7 (Tan et al., 2001) may recruit only those naïve T-cells that react with the vaccine antigen. Also, activation of co-stimulatory molecules by anti-OX-40 and anti-4-1BB Abs seems to be a promising approach as shown by Sharma et al. (2006). Since the adaptive immune system fails or is less effective at old age, activation of the innate immune system such as killer macrophages may be a useful alternative or addition to activation of the adaptive immune system. One way of activating killer macrophages is by a lipophylic glycopeptide L-TMP-PE (liposyl muramyl phosphatidylethanolamine; Nardin et al., 2006). Elimination of CD4+CD25-FOXP3+ T-cells (Nishioka et al., 2006) or CD8+CD28- T-cells (Filaci et al., 2004) may enhance vaccine efficacy at older age.

Also approaches that may improve vaccine efficacy in general are important. For instance, multi-antigen vaccines may prevent escape of genetically unstable tumor cells that have lost antigen expression. Many tumors produce lymphokines or factors that may inhibit vaccine-induced T-cells responses such as TGFβ, IL-6, COX-2 and its products PGE_2, PD1-ligand, or IDO (Gajewski et al., 2006; Park et al., 2004). Reduction of these factors may enhance vaccine efficacy as well. Also macrophages may produce these factors such as IL-6, IL-10, PGE_2, or TGBβ. Therefore, elimination of macrophages that produce IL-6, TGFβ, PGE_2 or IL-10 may improve vaccine efficacy. Improved delivery systems resulting in improved expression of the vaccine antigen in vivo, not discussed in this review, will certainly improve vaccine efficacy in general.

Prevention or elimination of metastases deserves more attention. In most cancers, metastases and not primary tumors contribute to morbidity and mortality. In contrast to primary tumors, metastases cannot be removed by surgery or radiation,

and are usually chemoresistant (Pardal et al., 2003). It is therefore encouraging that vaccines, such as Mage and Survivin, proved especially effective against metastases. Further improvement of vaccines against metastases may dramatically improve the clinical outcome of cancer treatment.

Finally, it is obvious that in most of the vaccine studies discussed here, immunological responses were analyzed in old (and young) mice without tumors, while the primary tumors are crucial in the development of vaccine-induced immune responses. As discussed earlier, primary tumors may inhibit vaccine-induced immune responses, may lose antigen expression in vivo and therefore escape vaccine-induced immune responses. Tumors may also stimulate macrophages resulting in the production of factors that may inhibit vaccine-induced immune responses. It is also obvious that none of the vaccine studies in which vaccination was tailored to old age, and despite good anti-tumor responses, activation of CD8 T-cells was shown in mice with tumors. Our own results with the 4T1 model did not show any CD8 response, while a significantly lower number of metastases was observed. Preliminary results suggest that the innate rather than the adaptive immune response was activated by vaccination. All together, we have little information about which immune cells really may have contributed to reduced tumor growth and improved tumor protection at older age. This brings us to the question why preclinical studies always look more promising than the eventual human clinical trials. For this reason, mice have been criticized as a good model for preclinical testing of cancer vaccines. However, analysis of the cancer vaccine studies discussed here demonstrate that mouse tumor models are good preclinical models for testing cancer vaccines, but that the design of preclinical studies in mouse tumor models in order to obtain successful results often do not reflect the situation in cancer patients, and may lead to wrong interpretations. Examples are (1) studying vaccine-induced immune responses in mice without tumors only, (2) applying vaccination when tumors are very small, (3) testing (foreign) human antigens, in mice; and last but not least (4) the ignorance of the age factor in human clinical trials.

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Metabolic Syndrome

Insulin Resistance, Chronic Inflammation and the Link with Immunosenescence

Dawn J. Mazzatti, Kavita Karnik, Radu C. Oita and Jonathan R. Powell

Contents

Abstract: Ageing is associated with an activation of the innate immune system which manifests in a chronic, low-grade, inflammatory status common in elderly individuals. Age-related inflammatory activity, as measured by increased serum levels of proinflammatory cytokines and activation of inflammatory signalling pathways, leads to long-term tissue damage and is thought to contribute to—and occur as a consequence of—immunosenescence. In addition to immune system deregulation, this elevated inflammatory status is associated with a number of age-related diseases and conditions, including neurodegeneration, atherosclerosis, sarcopenia, and diabetes, and is a main contributor to the age-related decline in physical function and vitality known as frailty. Inflammation is also an important component of the insulin resistance syndrome. In addition to age, a major risk factor for the development of the insulin resistance syndrome is obesity. Obesity is associated with increased proinflammatory cytokine production and altered regulation of both pro and antiinflammatory molecules, including a class of adipose-derived signalling molecules termed adipocytokines. The increased production of inflammatory mol-

D. J. Mazzatti (✉) · K. Karnik · R. C. Oita · J. R. Powell
Unilever Corporate Research
Colworth Park, Sharnbrook, Bedfordshire
United Kingdom MK44 1LQ
Tel.: +44 1234 222372; Fax: +44 1234 248010
E-mail: Dawn.Mazzatti@unilever.com

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ecules in obese and nonobese insulin resistant elderly individuals may contribute to age-related decline in health, including dysfunction of the immune system. Anti-inflammatory strategies for the treatment of the insulin resistance syndrome may promote remodelling of the immune system thereby contributing to remediation of immunity and prevention of frailty in the elderly population.

Keywords: Ageing • Inflammation • Insulin resistance • Immunosenescence • Frailty • Adipocytokines

1 Introduction

Robust immune and inflammatory responses are critical for survival. According to the antagonistic pleiotropy theory of ageing, it is suggested that whilst acute responsiveness of the immune system to challenge is required for tissue repair and resistance to infection, chronic inflammatory responses, a key feature of the ageing immune system, contribute to polarisation of cell-mediated immune responses and metabolic deregulation in advancing age. This immune dysfunction manifests in insulin resistance and contributes to development of degenerative diseases associated with ageing including Type 2 diabetes, cardiovascular and neurodegenerative diseases, and conditions associated with increased morbidity such as frailty.

1.1 Overview of the Immune System

The immune system is a complex network of lymphoid organs, cells, and soluble mediators, all of which act in a coordinated manner for host defence. The immune system is comprised of 2 functional nodes, the innate or native and the acquired or adaptive system. Cells acting in innate immunity not only include lymphoid cells such as neutrophils, monocytes, macrophages and natural killer cells, but many nonimmune cells such as fibroblasts, endothelial cells, and adipocytes also produce mediators and/or express surface markers allowing them to play a role in innate immunity. In contrast, the adaptive immune response mediates recognition of antigens and formation of antigen-specific memory cells capable of rapid activation and proliferation upon reexposure to the antigen. T- and B-lymphocytes are the major cellular components of the acquired immune system.

One of the major actions of the immune system involves communication by direct cell-to-cell contacts involving adhesion and signalling molecules. This is accomplished via production of chemical messengers such as cytokines, which modulate inflammatory and immune responses produced by a variety of cell types. Cytokines are involved in all aspects of the immune response and play a major role in directing the type of immunity generated in response to immune challenge. These mediators have been divided into several groups including interleukins, growth factors, and

chemokines. Cytokines include families such as the tumour necrosis factor (TNF) family and interferons.

In addition to mediating immune responses to challenge, cytokines also induce metabolic responses including induction of fever and loss of appetite. Inflammation is an integral part of this response. However, failure to resolve inflammatory responses after recovery or following completion of repair can be detrimental for health and has been implicated in the aetiology of inflammation-related diseases and conditions such as psoriasis, rheumatoid arthritis, atherosclerosis and cardiovascular disease.

1.2 Immunosenescence and Inflamm-ageing

Deregulation and deterioration of various components of the immune system—or immunosenescence—occurs in ageing. This loss of immunity results in increased incidence and severity of infectious disease, cancer and autoimmunity and contributes to enhanced morbidity and mortality in the elderly population (Pawelec and Solana 1997; Effros et al. 1997; Lesourd 1999). One important consequence of—and contributor to- the dysfunctional immune response in ageing is manifestation of chronic, low level inflammation, due to deregulation and overexpression of proinflammatory cytokines, a condition termed inflamm-aging (Franceschi et al. 2000). Since chronic, low-level inflammation is thought to be a risk factor for age-related disease and frailty, controlling inflammatory status in elderly individuals may provide a route to successful ageing. Recent reports have supported this hypothesis by demonstrating that individuals who are genetically predisposed to produce low levels of pro-inflammatory cytokines or high levels of antiinflammatory cytokines have an increased probability to reach extreme longevity (Bonafe et al. 2001; Lio et al. 2001; Giacconi et al. 2004; Van Den Biggelaar et al. 2004).

Several immune cell types are responsible for secreting cytokines in the inflammatory response. Type 1 T-cells (Th1) generate IL-2 and IFN-γ and contribute to cell-mediated responses whilst Type 2 cells are responsible for Th2 immunity by producing cytokines such as IL-4 and IL-5 leading to antibody responses. Monocytes and macrophages also produce Th1 and Th2 cytokines. Data from murine and human studies suggest that a switch from Th1 to Th2 immunity in ageing is an important contributor to immunosenescence (Cakman et al. 1996; Miller and Stutman 1981; Rink, Cakman and Kirchner 1998]. The general age-related decline in Th1 function is thought to mediate the shift towards Th2 immunity and may partly contribute to the increased incidence of inflammation-related diseases associated with ageing. Macrophages are an important contributor to this shift. A significant increase in Th1 and Th2 cytokines including the proinflammatory molecules TNF-α and IL-6 occurs in stimulated macrophages from elderly compared with young donors (Cossarizza et al. 1997). Secreted levels of IL-6 in the elderly are often high enough to be detectable in serum under unstimulated conditions and in the absence of an inflammatory response (Ershler et al. 1993), suggesting that low-level chronic inflammation is detectable in elderly humans. This increase in pro-inflammatory mediators such as

IL-6 and TNF-α may play a role in development of multiple age-related diseases including atherosclerosis, rheumatoid arthritis, fibrosis and dementia.

1.3 Inflammation and the Insulin Resistance Syndrome

Heightened inflammatory status directly contributes to a variety of poor ageing outcomes. In addition to low level inflammation resulting from—and contributing to—immunosenescence, chronic activation of innate immunity is a hallmark of the insulin resistance syndrome. In fact, current levels of inflammation predict progression of diabetes and future morbidity (Spranger et al. 2003). Chronic inflammation drives age-associated reductions in muscle mass (sarcopenia) which compounds insulin resistance (Barbieri et al. 2003). Increased inflammatory status leads to elevations of the antiinflammatory suppressor of cytokine signalling (SOCS) family with age (Peralta et al. 2002). These proteins have the potential to directly inhibit insulin pathways, contributing to insulin resistance and obesity.

Insulin regulates the uptake, oxidation, and storage of fuel in insulin-sensitive tissues such as skeletal muscle, liver, adipose tissue, and macrophages. Obesity is associated with resistance to the effects of insulin and can lead to diseases including Type 2 diabetes. Systemic chronic inflammation has been proposed to have an important role in the aetiology and pathogenesis of obesity-related insulin resistance. Population studies have demonstrated a correlation between proinflammatory cytokine production and metabolic deregulation. Bio-markers of inflammation including TNF-α, IL-6 and C-reactive protein (CRP) are present at increased levels in individuals who are insulin resistant and obese and increased production of these cytokines in individuals with the insulin resistance syndrome correlates with increased risk of developing type 2 diabetes mellitus and cardiovascular disease (Spranger et al. 2003; Pischon et al. 2007). In addition, is hypothesized that insulin resistance together with associated hyperinsulinemia, hyperglycemia, and heightened inflammatory cytokine production, might lead to a state of vascular inflammation and promote the development of atherosclerotic cardiovascular disease.

Hotamisligal and colleagues first reported a link between obesity, increased expression of proinflammatory TNF-α and reduced insulin action (Hotamisligal et al. 1993). They demonstrated that adipocytes derived from obese rodents directly secrete TNFα and hypothesized that inflammation was a contributor to the development of obesity. These observations were subsequently confirmed in humans, and it was further observed that weight loss in obese subjects corresponded with decreased TNF-α production (Kern et al. 1995). Several proinflammatory cytokines (including IL-6), suppressor of cytokine signalling proteins (SOCS), endoplasmic reticulum (ER) stress, IKKB and JNK signalling pathways have all been all been associated with developing insulin resistance (Rui et al. 2002; Croker et al. 2003). These studies demonstrate a link between adipose-produced cytokines, the immune system and insulin resistance. It is hypothesized that insulin resistance may

be accelerated—or initiated by—an innate immune response in which increased Th1 and Th2 cytokines such as TNF-α and IL-6 and proinflammatory adipose-derived cytokines such as leptin and resistin are expressed in adipose tissue of obese individuals.

1.4 Insulin Resistance in Ageing

In addition to obesity-induced insulin resistance, age itself is a risk factor for insulin resistance syndrome even in the absence of obesity. Compared to young individuals, normal weight, healthy elderly people have a marked tendency toward insulin resistance (Petersen et al. 2003). This obesity-independent insulin resistance is associated with reduced insulin-stimulated muscle glucose metabolism, impaired mitochondrial function, increased fat accumulation in tissues such as muscle and liver, and a general increase in adipose mass concomitant with decreased fat-free mass. Whilst it is known that a decrease in lean body mass and increase in fat mass occurs with ageing (Bosy-Westphal et al. 2003; Bartali et al. 2002) the mechanisms by which fat accumulation in peripheral tissues as a function of age results in the insulin resistance syndrome are poorly understood. One hypothesis is that insulin resistance in elderly individuals may occur as a consequence of altered inflammatory environment produced by redistributed adipose tissue. This is supported by the facts that intra-abdominal fat accumulates more rapidly than total fat and muscle mass is lost. Since insulin resistance is associated with altered Th1 and Th2 responses—which occur as a function of age—it is possible that the age-related proinflammatory background impacts on insulin action and peripheral responses. Fig. 1 depicts the balance between acute and chronic inflammatory activation and the impact of age, obesity, insulin resistance and immunosenescence on shifting the balance from appropriate acute activation and subsequent resolution of inflammatory signalling towards the development of chronic inflammatory conditions.

2 Obesity, Insulin Resistance and Inflammation

The incidence of obesity and obesity-related conditions and diseases is markedly increasing in developed and developing countries alike. Obesity predisposes individuals to increased risk of developing diseases such as atherosclerosis, diabetes, nonalcoholic fatty liver disease, cancers, and immune-mediated disorders such as asthma (Wellen and Hotamisligal 2005; Calle and Kaaks 2004; Mannino et al. 2006). In addition to these recent associations between obesity, insulin resistance, and disease, research in the past several years has identified multiple biological signalling pathways that link altered metabolic responses with a deregulated immune system (reviewed in Tilg and Moschen 2006). Many of these interactions between the metabolic and immune systems are linked via a network of soluble mediators derived from both cells

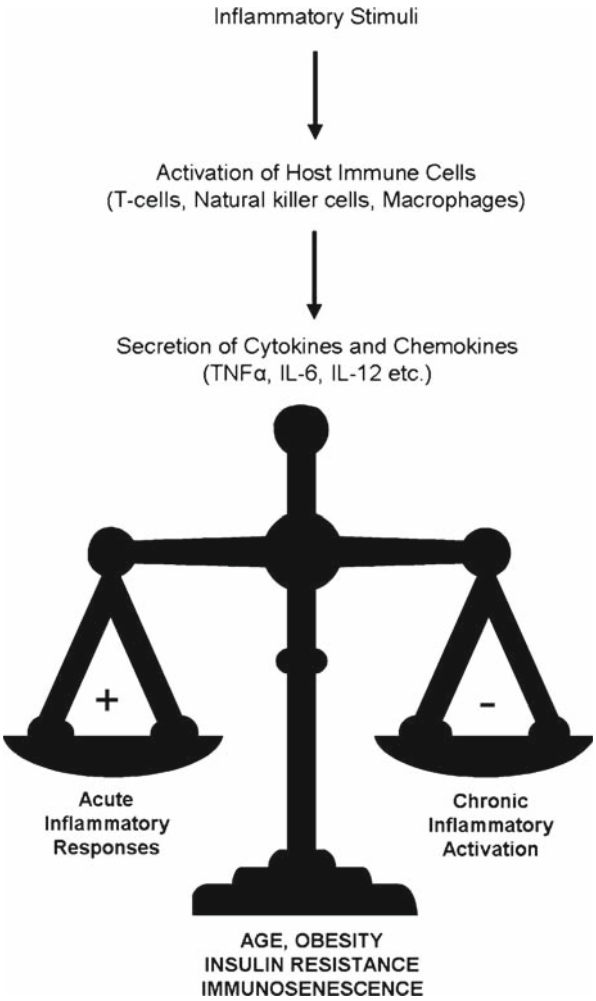

Fig. 1 Inflammatory stimulation and immune cell activation leads to both acute and chronic inflammatory responses

Inflammatory stimulation following injury or exposure to pathogen leads to activation of cell-mediated immunity. This activation leads to production of cytokines such as TNF-α and IL-6 which modulate immune responses and manifest in inflammation. Failure to resolve acute inflammatory responses after recovery or following completion of repair leads to chronic low level production of inflammatory cytokines. In conditions of ageing, obesity, insulin resistance and immunosenescence acute inflammatory processes may not be resolved resulting in chronically elevated inflammatory cytokine production.

of immune origin—such as macrophages—and cells in adipose tissue (adipocytes). These include immune cytokines such as TNFα, IL-6, IL-1, CC-chemokine ligand 2 (CCL2) and cytokines produced mainly by adipose tissue—termed adipocytokines or adipokines—such as adiponectin, leptin, resistin, and visfatin. The roles of these

cytokines and adipocytokines in obesity, insulin resistance and their impact on chronic inflammation will be discussed in the following sections.

2.1 Adipose Tissue as a Regulator of Immune Response

Obesity is associated with a chronic inflammatory response which is characterized by abnormal cytokine production, increased synthesis of acute-phase reactants—such as C-reactive protein (CRP)—and the activation of proinflammatory signalling pathways (Wellen and Hotamisligal 2005). Whilst it is known that proinflammatory pathways are activated in adipose tissue of obese individuals, whether the cytokines are produced locally by adipocytes or by circulating macrophages or other immune cells present in adipose tissue is as yet unclear. In 2003, 2 papers demonstrated that diet-induced obesity is associated with infiltration of macrophages into white adipose tissue (Weisberg et al. 2003; Xu et al. 2003). Infiltrated macrophages, a component of the stromal vascular fraction of adipose tissue, are responsible for production of proinflammatory cytokines. Since the adipose tissue of obese individuals is infiltrated with macrophages it is possible that these macrophages account for at least some of the soluble mediators in adipose tissue. In fact, macrophages appear to be the main source of TNFα in obese individuals, whilst adipocytes contribute almost one third of the circulating IL-6 in obese patients [Fantuzzi et al. 2005], suggesting that cells of both immune and adipose origin contribute to inflammation and insulin resistance in obesity. Adipose-produced CCL2 (also known as MCP-1) has recently been implicated as a potential recruiter of macrophages to adipose tissue in states of obesity (Kanda et al. 2006). The interaction between macrophages and adipocytes in adipose tissue may perpetuate a vicious cycle of macrophage recruitment and production of proinflammatory cytokines. Importantly, the development of adipocyte insulin resistance has been closely linked to infiltration of macrophages (Weisberg et al. 2003; Xu et al. 2003). However, if and how the entry of macrophages into white adipose tissue might lead to systemic insulin resistance remains unclear. However, it is believed that altered secretion of adipocytokines by adipose tissue during obesity may play an important part of pathogenesis of insulin resistance.

An important adipocytokine produced by adipocytes, skeletal muscle cells, cardiac myocytes and endothelial cells, is adiponectin. Adiponectin expression correlates inversely with insulin resistance as serum levels are markedly reduced in individuals with visceral obesity and insulin resistance (Arita et al. 1999). TNF-α and IL-6 are major regulators of adiponectin levels as both proinflammatory cytokines suppress transcription of adiponectin (Maeda et al. 2002; Fasshauer et al. 2003). Positive regulators of adiponectin include weight loss [Bruun et al. 2003] and activation of the peroxisome proliferator-activated receptor-γ (PPARγ), by either its natural ligands, arachidonic acid-metabolites such as 15-deoxy-Δ-12,14,-prostaglandin J2 (Forman et al. 1995) or pharmacological ligands thiazolidinediones (Maeda et al. 2001; Iwaki et al. 2003), which are important pharmaceutical drugs for the treat-

Table 1 Adipocytokines and their proposed effects on innate immunity

Adipocytokine	Downstream signalling targets	Effect on inflammatory response
Adiponectin	NFκB, TNFα, IL-6, IL-10, PPARα, IL-1RA	Antiinflammatory
Leptin	TNFα, IL-6, IL-12,	Proinflammatory
Resistin	NFκB, TNFα, IL-6, IL-12	Proinflammatory
Visfatin	IL-6, IL-8	Not determined

ment of type 2 diabetes mellitus. Adiponectin has been found to suppress inflammation in various animal models and can suppress macrophage activity not only in adipose tissue but also in liver.

In opposition to the insulin sensitising and antiinflammatory properties of adiponectin, another adipocytokine, leptin, is considered to be a proinflammatory cytokine due to its structural similarity to other proinflammatory cytokines such as IL-6. The opposing functions of adiponectin and leptin and other adipocytokines on inflammatory responses are summarized in Table 1. In addition to their roles in inflammation, each of these adipocytokines has also been associated with insulin resistance and Type 2 diabetes, providing a link between innate immunity and insulin responses. The role of leptin in modulating the immune response and inflammation has become increasingly apparent and has been reviewed recently (La Cava and Matarese 2004; La Cava et al. 2003). In addition to its role in regulating neuroendocrine function, energy homeostasis and hematopoiesis, leptin has also been shown to be an important regulator of immune-mediated diseases and inflammatory processes (La Cava and Matarese 2004). Although the main function of leptin is in control of appetite, mice with mutations in the gene encoding leptin (*ob/ob* mice) or the gene encoding the leptin receptor (*db/db* mice) are obese and have various defects in cell-mediated and humoral immunity such as altered T-cell responses (Mandel and Mahmoud 1978; Lord GM et al. 1998).

2.2 Links Between Lipid Metabolism and Inflammation

Whilst it is increasingly recognized that obesity is characterized by chronic activation of inflammatory molecules (Kahn and Flier 2000; Wellen and Hotamisligil 2003; Wellen and Hotamisligil 2005), the fundamental mechanisms responsible for activation of inflammatory pathways in obesity are poorly understood. Elevated levels of free fatty acids (FFAs) in obesity have been suggested to cause insulin resistance due to increased release of FFAs from adipose and subsequent entry into circulation. Release of FFAs in this manner due to deregulated adipose tissue leads to impaired ability of insulin to suppress hepatic glucose production and to stimulate glucose uptake into skeletal muscle (Lam et al. 2003; Boden et al. 2005; Dresner et al. 1999). Intracellular mechanisms by which FFAs cause insulin

resistance are starting to emerge and include kinases linked to inflammatory signalling (protein kinase C, IκKα, and c-Jun N-terminal kinase (JNK)) (Lam et al. 2003; Boden et al. 2005; Dresner et al. 1999). However, the sensing mechanisms by which FFAs activate intracellular inflammatory signalling, which then induce insulin resistance, are unclear.

2.2.1 Toll-like Receptors (TLRs)

Mammals defend themselves against tuberculosis and other microbial diseases in part through activation of TLRs which initiate innate immune responses. TLRs are a family of pattern-recognition receptors that activate proinflammatory signalling pathways in response to microbial pathogens (Medzhitov 2001). TLR4, the best characterised TLR, binds to the lipopolysaccharide component of the bacterial cell wall and initiates interactions which ultimately result in activation of the nuclear factor κB (NFκB) signalling pathway. Activated NFκB transcriptionally regulates cytokines, chemokines, and other effectors of the innate immune response (Zuany-Amorim, Hastewell and Walker 2002). FFAs have been demonstrated to utilise the innate immune receptor TLR4 to induce proinflammatory cytokine expression in macrophages, adipocytes, and liver [Shi, Kokoeva, Flier et al. 2006]. Shi et al. further demonstrated that TLR4 signalling was required for FFA-induced insulin resistance in adipocytes suggesting that TLRs are involved in initiating inflammatory responses in response to dietary lipids. These data are the first to provide a link between the innate immune system and metabolic responses and suggest that TLR4 may be at the crossroads of processes that regulate insulin resistance and chronic inflammation.

2.2.2 Fatty Acid Binding Proteins (FABPs)/Receptors

Although insulin resistance in response to lipid infusion was found to be attenuated in TLR4-deficient mice, the effects were not completely abolished and perhaps only slowed in progression, suggesting that additional mechanisms may be involved. One potential mechanism for TLR-independent lipid-induced insulin resistance is through regulation of inflammatory responses mediated by FABPs and their receptors. FABPs may contribute to the development of insulin resistance in response to dietary lipids. In addition to their role in the regulation of energy balance and obesity, evidence obtained recently indicates that FABPs may serve as master regulators in the control of cellular inflammatory responses through regulation of inflammatory mediators JNK and PPARγ (Hirosumi et al. 2002; Gao et al. 2002) thereby establishing a relationship between cellular stress responses, inflammatory cytokine production and obesity. Firstly, adipose-specific FABP4 (or a-FABP) may coordinate the lipid-mediated activation of stress kinases such as JNK or IκK under immune or metabolic stimuli thus linking lipid signalling to proinflammatory signalling and antiinsulin action [Hotamisligal et al. 1996]. It

has been hypothesized that the presence of FABP4 in periods of feast and famine may have been beneficial to maintain both a strong immune response and adequate fuel reserves in adipose tissue in fitting with the concept of "thrifty" phenotype to survive [Auwerx et al. 2003]. However, with increasing prevalence of excessive caloric intake, decreased energy expenditure and high stress lifestyle, FABPs may not be able to maintain inflammatory or metabolic homeostasis and the presence of these proteins may actually aid in the formation of obesity, dyslipidemia and inflammatory responses [Hotamisligal et al. 1996]. This may also occur as a function of age. Combined with the prevalent increased proinflammatory IL-6 production in elderly individuals, these alterations in inflammatory homeostasis have serious and detrimental implications for health in ageing populations.

2.2.3 Peroxisome Proliferator-activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family. PPARs function primarily as regulators of lipid/lipoprotein metabolism and glucose homeostasis, but also influence cellular proliferation, differentiation, and apoptosis. The PPAR family members are expressed in a tissue-specific manner: PPARα is highly expressed in oxidative tissues such as liver, muscle, kidney and heart and in cells involved in the immune responses including endothelial cells, monocytes, macrophages and lymphocytes. In contrast, although PPARγ is expressed in immune and endothelial cells, the molecule is predominantly expressed in intestine and adipose tissue. In addition to synthetic thiazolidinediones, fatty acids and eicosanoids are natural PPAR ligands suggesting not only a metabolic role for PPARs, but function also in inflammation control. This hypothesis is supported by the facts that (1) PPARα deficient mice display a prolonged inflammatory response and (2) PPAR activators have been shown to inhibit the activation of inflammatory response genes by negatively interfering with NFκB, STAT and AP-1 signalling pathways.

PPARα is thought to regulate inflammatory pathways mainly through inhibition of inflammatory gene expression. Hepatic PPARα activation has been repeatedly shown to reduce hepatic inflammation in a variety of stress-induced models. The immunosuppressive effects of PPARα includes interference with several proinflammatory transcription factors including signal transducer and activator of transcription (STAT), activator protein-1 (AP-1), and NFκB (Delerive et al. 1999). PPARα can also inhibit cytokine signalling pathways via down-regulation of the IL-6 receptor (Gervois et al. 2004) and up-regulation of sIL-1 receptor antagonist (Stienstra et al. 2007). Importantly, PPARα expression is known to decrease with age in liver, kidney, and heart, suggesting that the beneficial effects of PPARα on limiting inflammatory responses are lost in ageing and may contribute to chronic inflammation observed in elderly individuals. Ligand-induced activation of PPARα or PPARγ in macrophages can effectively inhibit activation-induced inflammatory cytokine production (Padhilla et al. 2000; Harris and Phipps 2001). PPARα also

functions in other immune cells including T-lymphocytes (Jones, Manning, and Daynes 2002).

Although PPAR expression is under the control of a wide variety of factors, inflammatory cytokines such as TNFα, IL-1, and IL-6 have been shown to decrease PPARγ expression in adipocytes (Tanaka et al. 1999). In contrast, in monocytes and macrophages, the anti-inflammatory molecule IL-4 induces PPARγ expression (Huang, Welch, Ricote et al. 1999).

Taken together, it has become quite clear in recent years that PPARs play critical roles in the regulation of energy homeostasis (through regulation of lipid and carbohydrate metabolism). Dysregulated PPAR activity has been described in a number of pathological states including cancer, inflammation, infertility, demyelination and atherosclerosis (Devchand et al. 1996; Mueller et al. 2000; Berger and Moller 2002; Takano and Komuro 2002). The expression of PPARs in both myeloid and lymphoid cell lines suggests a link between PPARα expression and the immune system. Due to the ability of PPARα in particular to act in immune responses and in metabolic functions, the ability to regulate PPAR activity may represent a useful therapeutic strategy to treat diverse pathological conditions, including inflammation- and obesity-related diseases.

3 Impact of Chronic Inflammation and Insulin Resistance on Frailty and Disability

Several recent studies suggest that immunosenescence may contribute to the decline of physiological functions that occur in ageing. First, in ageing, the immune system becomes less able to respond to cues from the internal and external "environment" (Pawelec, Hirokawa and Fulop 2002). Chronic antigenic stress that occurs in ageing contribute to clonal expansions of CD4+ memory T-cells which fill the "immunological space" leaving relatively few naïve, responsive cells to provide defence against challenge. In humans, common, persistent viral infections such as CMV contributes markedly to the persistent clonal expansions commonly seen in the elderly (Khan et al. 2002; Ouyang et al. 2003; Hadrup et al. 2006). Moreover, the absolute number of accumulated cells is an important part of the "Immune Risk Profile" (IRP) predicting mortality in longitudinal studies of the very elderly (Wikby et al. 2005). These accumulated cells are dysfunctional, and may not only be filling the available "immunological space" but may be actively suppressive of responses of other clones (Pawelec et al. 2006). These age-related changes in the immune system manifest a remodelling of immunity accompanied by chronic low level increase in proinflammatory cytokines. These inflammatory markers are more prevalent in frail elderly subjects and appear to be caused by both chronic antigenic stress and oxidative damage and are associated with increased risk of developing multiple age-related degenerative diseases (Franceschi et al. 2000; Ginaldi 2005).

The frailty syndrome is a complex clinical condition that is somewhat ambiguous in definition. Many definitions of frailty have been proposed in the literature. Recently, Fried and colleagues have attempted to clarify these definitions are have highlighted the importance of several markers of the frail phenotype. These include wasting (muscle, strength, weight loss) and reductions in endurance, balance and mobility (Fried et al. 2001). In addition, decreased cognitive performance is generally considered an important component of the frailty phenotype. The biological contributors to frailty include sarcopenia (loss of muscle mass and strength), neuroendocrine decline, and immune dysfunction including heightened production of proinflammatory markers.

The involvement of the immune system in the pathogenesis of age-related decline and in progression of frailty has been hypothesized for a number of years due to the link between increased proinflammatory cytokine production in ageing (particularly IL-6) and the relationship between IL-6 with physical function and disability. Several studies have consistently demonstrated that increased levels of IL-6 and other markers of inflammation are associated with risk of physical disability in late life (Cohen et al. 1997; Ferrucci et al. 1999). The catabolic effects of pro-inflammatory cytokines on muscle may have a direct effect on loss of muscle mass in ageing, thereby contributing to the frailty phenotype. In addition, the synergistic effect of hormones, insulin signalling, and immune changes in ageing may also underlie the pathogenesis of frailty and physical disability.

4 Intervention Strategies Targeted to Reduce Chronic Inflammatory Responses

4.1 Pharmaceutical Interventions

Pharmaceutical interventions aimed to limit chronic inflammatory activation through targeting innate immunity have the potential benefits in reducing the risk of age-related diseases and conditions including frailty. In animals, the neutralization of TNF-α by injected monoclonal antibodies not only reduced inflammatory TNF-α production but improved insulin sensitivity (Hotamisligil, Shargill and Spiegelman 1993; Paquot 2000; Ofei 1996; Shoelson, Lee and Yuan 2003). However, this effect was not observed in humans and patients on anti-TNF-α therapy became more susceptible to bacterial infections (Estrach et al. 2004) demonstrating the complexity of the innate immune system and problems associated with an unbalanced immune response. Therapeutic studies demonstrating health benefits using high-dose aspirin and salicylates in Type 2 diabetes patients support a role for inflammation in metabolic disease (reviewed in Shoelson, Lee and Goldfine 2006). In addition to reductions in systemic inflammation, decreases in CRP levels and reductions in fasting blood glucose and serum triglycerides were also observed following these anti-inflammatory therapies (Shoelson et al. 2003). In addition, inhibition of cycloxygenase by NSAIDS (nonsteroidal antiinflammatory drugs) constitutes a readily-used clinical approach to treat inflammatory

conditions. Certain NSAIDS, including indomethacin and ibuprofen, are activators of PPARγ and PPARα in the micromolar range (Lehmann et al. 1997) and as such represent important metabolic and inflammatory treatments.

Immunotherapy of chronic inflammatory conditions is in the early stages of development. Due to the link with nutrition and immunity, leptin is an attractive target for immunotherapeutic approaches that reduce its proinflammatory effects. Leptin is a relatively new candidate gene target for immune therapies, in particular the suppression of autoimmune responses. Leptin-based therapies are currently only administered to individuals with genetically-based leptin deficiency or to extremely obese non-leptin deficient patients. In addition to reductions in food intake and obesity, leptin-based therapies also restore neuroendocrine, reproductive, and immune functions (Farooqi et al. 1999; Farooqi et al. 2002). Immunoregulatory functions of leptin treatment include increase in thymic output of T-cells and restoration of Th1 responses (Lord et al. 1998), which may provide additional benefits in the context of ageing. Given the strong proinflammatory effects of leptin, abrogation of its activity with administration of antibodies to leptin, its receptor or soluble recombinant leptin-receptor (which reduces circulating levels of leptin) may reduce chronic inflammation. Caloric restriction, diets rich in n-3 polyunsaturated fatty acids (fish oils) or low in saturated fatty acids could also decrease circulating leptin levels with little effect on body weight, an important consideration in ageing individuals. In contrast to promising effects of leptin-based therapies, despite optimism and a concerted effort to produce TLR-based pharmaceutical drugs, few studies have been conducted demonstrating that attempts to target TLR and the innate immune response can successfully treat human conditions (Fasciano and Li 2006; Ulevitch 2004).

Thiazolideinediones (TZDs) such as rosiglitazone and piogliazone are synthetic PPAR-γ agonists used clinically to treat Type 2 diabetes due to their insulin sensitizing properties. In the last several years, PPARγ has been implicated as a regulator of the cellular inflammatory response and as such, PPARγ agonists such as TZDs may exert their antiinflammatory effects by negatively regulating the expression of proinflammatory genes induced during macrophage differentiation or activation including IL-1β, IL-6 IL-12, TNFα, and IFNγ (von Knethen and Brune 2003; Jiang, Ting, Seed 1998). The therapeutic effects of PPAR-γ ligands exceed their insulin-sensitising actions and exert multiple beneficial effects in conditions associated with insulin resistance and inflammation. Multiple in vivo studies have demonstrated that glitazones exert potent antiinflammatory effects in both acute and chronic inflammatory settings. However, several recent studies have found that inflammatory stimuli independently induce PPAR-γ expression in immune cells, but not in nonactivated monocytes/macrophages (Leininger, Portocarrero, and Houseknecht 1999; Ricote et al. 1998), suggesting that during inflammatory processes PPAR-γ expression may be differently modulated in a cell-type-specific manner depending on type of inflammatory challenge and signalling pathway activated. Taken together, these data suggest that while PPAR-γ agonists may be useful in the therapy of some chronic inflammatory disorders such as psoriasis, the potential impact of PPAR-γ agonists on immunosenescence may be more complex.

4.2 Physical Activity

It is accepted that macrophage infiltration and inflammation of adipose tissue in states of obesity and consequent low grade inflammation is involved in the pathogenesis of the metabolic syndrome and Type 2 diabetes. Therefore strategies targeted to weight reduction, increase muscle mass, and shrinkage of adipose tissue also focus on attenuation of inflammation. The immunomodulatory effects of exercise are well documented including a shift in the production of Th1 and Th2 cytokines, enhanced NK-cell and T-cell activity, improved antibody responses (reviewed in Moyna et al. 1996; Venjatraman, Fernandes 1997; Shephard, Shek 1995; Mazzeo 1994; Shinkai, Konishi, Shephard 1998). Exercise and weight loss have direct anti-inflammatory effects on the innate immune system and on adipose tissue. In addition to weight reduction, moderate exercise is associated with many beneficial effects on degenerative diseases of ageing including reduced all-cause mortality (Blair and Wei 2000), improved metabolic syndrome (Lakka et al. 2003) and cardio-respiratory fitness (Dunn et al. 1999; Wei et al. 2000). Furthermore, human exercise intervention studies show a causal reduction in inflammatory markers including CRP (Church et al. 2002; Ford 2002). It is widely acknowledged that moderate exercise exerts many health benefits with no contraindications if prescription is tailored to an individual's health status.

4.3 Nutrition

Dietary interventions in old mice have demonstrated that activation of PPARα by dietary supplementation including vitamin E in combination with DHEA or molecular activator WY14, 643 resulted in potent in vivo inhibition of inflammation. In these studies activated PPARα up-regulates the synthesis of Iκβ and inhibits the activity of NFκβ, thereby regulating the inflammatory process (Delerive et al. 1999, 2000). Not only are DHEA or WY14,643 supplementation strategies effective at suppressing active NFκβ in lymphoid tissues, they also correct the abnormal expression of the various proinflammatory molecules that are over-produced in ageing (Poynter and Daynes 1998). When similar experiments were conducted in aged PPARα -/- mice, supplementation was found to be ineffective, demonstrating that modulation of PPARα is necessary to promote the antiinflammatory properties of vitamin E (Spencer et al. 1997).

Recent studies have suggested that n-3 fatty acids, a major component of fish oil, may protect against high-fat diet-induced insulin resistance, perhaps through modulation of the post-prandial inflammatory response. In rats fed dietary fish oil instead of safflower oil, protection from fat–induced insulin resistance was observed (Jucker et al. 1999; Ikemoto et al. 1996; Storlien et al. 1987). The ability of fish oil to preserve insulin sensitivity is likely mediated by polyunsaturated (omega-3) fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic

acid (EPA). The ability of these fish oils to modulate insulin sensitivity is thought to occur through modulation of PPARα and subsequent decrease in intracellular lipid (Neschen et al. 2007). Studies in humans have shown some beneficial effects of n-3 fatty acids on metabolic profiles, including reduced production of inflammatory cytokines (Browning 2003; Browning et al. 2007) particularly in the context of drifting chronic inflammation with age (reviewed by Calder 2003; 2006). However, differential results were obtained depending on type of n-3 fatty acid ingested (eicosapentaenoic acid, docosahexaenoic acid, or linoleic acid). In addition, reduced lymphocyte proliferative responses and decreased phagocytic activity associated with increased n-3 consumption may occur in elderly populations (Rees et al. 2006). Thus, beneficial effects of n-3 fatty acids on age-related inflammation must be monitored carefully with the potential for reduced immunity.

There are clear associations between low serum zinc levels and compromised immune function (Rink and Gabriel 2000; Ibs and Rink 2004). Zinc deficiency in the elderly is very prevalent. In fact, the Third National Health and Nutrition Examination Survey (1988–1994) showed that only 51.1% of 51–71 year-old and 42.5% of >71 year-old elderly individuals had adequate zinc levels (defined as =/> 77% 1989 RDA (Briefel et al. 2000). Despite these figures, studies on zinc supplementation in the elderly have been discouraging, with either no benefit or even adverse affects reported following supplementation (Bogden et al. 1988; Chandra et al. 1993). These studies may be compounded by differential effects of dose and the elderly subpopulation studied. Evidence suggests that low (Girodon et al. 1999), but not high dose (Provincali et al. 1998) zinc supplementation improves vaccine responses in the elderly. It is likely that high doses–resulting in >30uM plasma zinc levels–most likely inhibit a range of T-cell functionalities (Cakman et al. 1997; Wellinghausen et al. 1997; reviewed by Ibs and Rink 2004).We have previously reported that in addition to its essential functions in growth and development, maintenance of the immune system, and as a cofactor for transcription and replication factors (Rink and Gabriel 2000, 2001) zinc may also act as a potent anti-inflammatory molecule by directly effecting the expression of a range of inflammatory mediators. Fig. 2 demonstrates the effects of zinc supplementation and deprivation on IL-6 and leptin mRNA expression in cultured Jurkat (T-cells) and THP-1 cells (monocytes). These data demonstrate that zinc deprivation—or corresponding low zinc status in the human ageing condition—may negatively impact on inflammatory gene expression and contribute to chronic low-level inflammation. In addition, we have recently obtained data suggesting that in selected individuals with elevated IL-6 production, zinc may reduce proinflammatory gene expression including modulation of IL-6, leptin, and FABP4 mRNA levels (Mazzatti et al. 2007). Taken together, zinc supplementation in specific sub-populations of zinc-sufficient elderly individuals or zinc-deficient individuals may restore of immunity and metabolic homeostasis as well as provide antiinflammatory benefits.

The discovery that the vitamin D receptor (VDR) is expressed by antigen presenting cells (APCs) such as macrophages and dendritic cells and also by lymphocytes following activation suggest a role for 1,25(OH)2D3 in the immune system [Mathieu

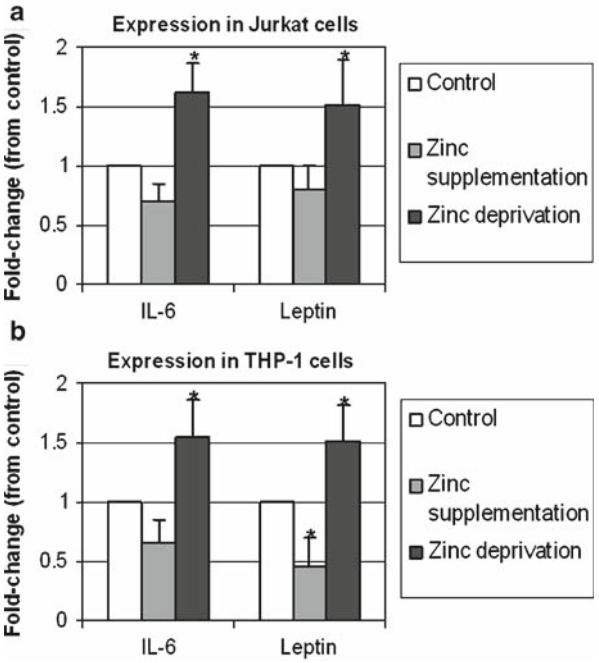

Fig. 2 Zinc supplementation and deprivation alters IL-6 and leptin mRNA expression in lymphocytes

Relative IL-6 and leptin mRNA expression in Jurkat (a) and THP-1 (b) cells following zinc supplementation (grey bars) and deprivation (black bars) compared to cells grown in normal growth media. Jurkat and THP-1 cells were plated at a density of 2×10^5 per ml in RPMI 1640 (Cambrex, Germany) supplemented with 10% FCS (PAA, Germany), 100 U/ml penicillin and 100µg/ml streptomycin. Cells were cultured for 40h at 37°C, 100% humidity and 5% CO2 either as untreated controls, or in the presence of either 50µM ZnSO4 (zinc supplementation), or 2.5µM of the membrane permeant zinc chelator TPEN [N,N,N',N'- tetrakis-(2-pyridylmethyl)ethylenediamine] (zinc deprivation). Total RNA was isolated using the Qiagen RNeasy kit (Qiagen, Germany). In vitro transcription was performed with the Superscript III First-Strand Synthesis System with random hexamer primers (Invitrogen, UK) and the Bio-rad I-Cycler (Bio-rad, CA, USA) was used for real time RT-PCR. Reactions were prepared using Platinum qPCR supermix with Taqman probes (FAM-490, Applied Biosystems, UK) for IL-6 (Hs00174131_m1), leptin (Hs00174877_m1), and GAPDH (Hs99999905_m1). PCR thermocycler conditions were 50°C for 2 minutes, 90°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All samples were run in triplicate with both test probes and the control gene human GAPDH to control for differences in amount of starting material. A standard curve was created for each PCR reaction. Fold-changes were calculated by normalizing the test crossing threshold (Ct) with the housekeeping control Ct (ΔCt) and calculating ΔΔCt by comparing treatment condition to untreated control. Values are shown as means from n=3 independent experiments ± S.D., values significantly different from the corresponding control ($p<0.05$, ANOVA) are indicated (*).

et al. 2002]. The enzyme responsible for the rate limiting hydroxylation step in the synthesis of 1,25(OH)2D3, 1-α hydroxylase, is expressed by activated macrophages. This enzyme is identical to the renal form but its expression is regulated differently. Renal 1-α hydroxylase is mainly regulated by mediators of calcium

and bone homeostasis (PTH and 1,25(OH)2D3 itself) while its macrophage version is under the control of immune signals such as interferon-γ. A paracrine role of vitamin D in the immune system is postulated based on the widespread presence of VDR in the different cell types of the immune system and 1-α hydroxylase activity regulated by immune signals.

Vitamin D affects T-cell function in several ways. Antigen-stimulated T-lymphocyte proliferation, cytokine secretion and cell cycle progression are inhibited by in vitro addition of 1,25(OH)2D3. Vitamin D directly affects the transcription of several key cytokines of Th1 lymphocyte such as IFN-γ and IL-2. By inhibiting IFN-γ transcription, 1,25(OH)2D3 prevents further antigen presentation to and recruitment of T-lymphocytes (Cippitelli et al. 1998). By inhibiting transcription of pro-inflammatory cytokines or influence Th1/Th2 responses, vitamin D may play an important role in the context of age-related chronic inflammation.

The link between vitamin D deficiency and type 2 diabetes has been known for many years (Gedik 1986). Receptors for 1,25-(OH)2D3 are found in β cells (Lee et al. 1994). Pancreatic β cells also contain vitamin D-dependent calcium binding protein, called as calbindin-D28k (Sooy et al. 1999). The expression of calbindin-D28k has been shown to protect β cells from cytokine-mediated cell death (Rabinovitch et al. 2001). Elimination of vitamin D deficiency has improved glucose tolerance in humans (Kumar et al. 1994; Gedik 1986; Boucher et al. 1995). The doses used range from 2000 IU/day to single intramuscular injection of 100,000 IU. In a recent analysis of Nurses Health Study, the relative risk of Type 2 diabetes was 0.87 comparing the highest with the lowest category of vitamin D intake from supplements (Pittas et al. 2006). However, supplementation of vitamin D to vitamin D sufficient patients with Type 2 diabetes or with impaired glucose tolerance have shown conflicting results. Borissova et al. (2003) have shown improvements but Isaia et al. (2001) did not report any effect suggesting that the beneficial effects of vitamin D on glycemic regulation may only present in individuals with vitamin D deficiency. However, further studies are needed to clarify these aspects.

5 Future Research Prospectus

Our understanding of the pathogenic role of inflammation and its contribution to age-related disabling conditions such as frailty is rapidly expanding. However, the biological mechanisms underlying—and/or contributing to—chronic activation of inflammatory pathways including immunosenescence and insulin resistance is less well understood. A comprehensive, systemic approach is needed to bridge this gap in knowledge. In particular systems biology-based approaches may aid in identifying the molecular contributors to the drift in inflammatory status in ageing. These may include factors which respond to environmental cues, including—but not limited to—damage/stress sensors and molecules involved in signalling and cell-to-cell communication including chemokines and cytokines. In addition,

since many cell types and organs are affected in ageing, it remains important to consider the global impact of these deregulated systems. To resolve this issue, systems-biology based applications must be used. Critically, in the context of chronic activation of inflammatory pathways, one must investigate the physiological actions and cross-talk between diverse cell types such as adipocytes, immune cells, stromal cells, epithelial cells, and fibroblasts. Understanding how these cells become deregulated and communicate differently in the context of ageing or in states of insulin resistance or obesity can only be resolved using systems biology approaches. Systems-based approaches will enable integration of previously collected data obtained at the organism, gene, protein and metabonome levels and will aid in addressing the complex relationships between age-related conditions such as frailty and immunosenescence with physiological disturbances such as heightened inflammatory status, adipose deregulation and insulin resistance. These approaches will also aid in identifying science areas where further targeted

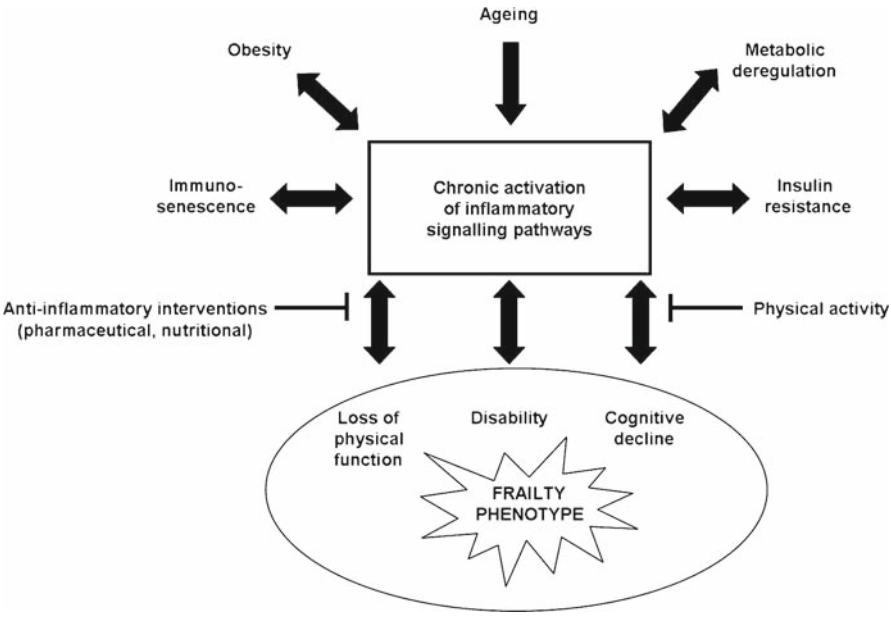

Fig. 3 Age-related chronic activation of inflammatory pathways contributes to the development of the frailty phenotype

Conditions such as ageing, obesity, immunosenescence, insulin resistance and states of metabolic deregulation contribute progressively towards the development of a chronic inflammatory state. In addition to the impact of these conditions on inflammation, elevated inflammatory cytokine production can also perpetuate and further exacerbate many of these conditions. Inflammatory status is a major risk factor that influences multiple components of the frailty phenotype including loss of physical function, cognitive decline, and disability. Physical activity, nutrition, and pharmaceutical agents are important modulators of inflammation and as such represent modes of intervention.

research is necessary to fill gaps in the knowledge base. Strategies to interpret these data with a evolutionary-based perspective may be particularly effective, according to the hypothesis that ageing—in particular immune ageing—is a process of maladaptive remodelling dominated by adaptation and response to inflammatory stimuli (Franceschi et al. 2007). This hypothesis is supported by the fact that the immune system provides robustness against pathogenic threats throughout the organism's lifetime yet it can also adversely affect the organism (as in autoimmune disease or in conditions of chronic activation of inflammatory pathways). Recent studies have started to emerge in which network-based approaches have been utilised to investigate the complexity of human immune and inflammatory responses (Calvano et al. 2005; Kitano and Oda et al. 2006). Taken together, these approaches may enable identification of new functional modules or nodes that are perturbed in disease states which may represent novel targets for immune intervention.

6 Concluding Remarks

In the last several years, the complex relationship between insulin resistance, ageing, and immune dysfunction has begun to evolve. A common mediator in the aetiology of each of these conditions is chronic inflammation (Fig. 3). Understanding the mechanisms that contribute to deregulated immune responses in ageing—and as a function of insulin resistance—may bring practical benefits in developing immune interventions for the elderly aiming to reconstitute appropriate inflammatory responses, restore insulin sensitivity, and prevent, delay or reverse the development of frailty and multiple age-related diseases. Systems biology-based applications will aid in this endeavour.

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Neurodegenerative Diseases

Decline of Immune Responsiveness: A Pathogenetic Factor in Alzheimer's Disease?

Elke Richartz-Salzburger and Niklas Koehler

Contents

1 Introduction

While the etiopathogenesis of Alzheimer's disease (AD) still remains unresolved, a growing body of evidence indicates the involvement of the immune system. Yet, both character and the significance of the observed alterations are matter of dispute.

During the seventies and eighties of the 20th century a high amount of literature accumulated dealing with the impact of immunological factors on neurobehavioral pathology associated with aging and AD (Richartz et al. 2004).

The putative relevance of inflammatory processes is shown by over 20 epidemiological studies suggesting a potential benefit of antiinflammatory intervention (Akiyama et al. 2000; McGeer and McGeer 1999). Further indication of a pathophysiological role of inflammation in AD is given by the presence of inflammatory mediators in the AD brain, including proinflammatory cytokines, acute phase proteins and the full complement cascade (Hüll et al. 1996; Mrak et al. 1995; Tarkowski et al. 1999). In summary, data available suggest that the AD brain undergoes chronic inflammatory process mediated by activated glial cells, targeted on the destruction of senile plaques, but lethal to surrounding neurons (McGeer & McGeer 2003).

The understanding that the brain is not that immunologically privileged site that it has been considered before is the result of modern psychoneuroimmunological research. There is an active and highly regulated communication between the

E. Richartz-Salzburger (✉) · N. Koehler
Department of Psychiatry and Psychotherapy
University of Tübingen
Osianderstrasse 24
DE-72076 Tübingen, Germany
E-mail: elke.richartz@med.uni-tuebingen.de

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brain and the immune system, and consequently, peripheral reactions can influence the cerebral immune response. Vice versa, cerebral immune processes can lead to peripheral immune alterations.

Against this background, numerous studies have been carried out focusing peripheral immunological alterations in AD.

In particular, the occurrence of brain—reactive autoantibodies in serum of patients with AD has raised the question of whether autoimmune processes could contribute to the clinical syndrome. Experimental animal studies have suggested a relationship between autoimmune status and age-associated cognitive decline (Richartz et al. 2004). In demented patients, serum autoantibodies against several self-antigens have been observed. However, the increase of autoantibody concentrations in the serum is not specific, but rather reflects age-dependant effects on the immune status of the patients (Schott et al. 1996, 1997).

Further studies did not confirm the presence of increased antibodies concentrations in AD. Antibodies against CD95 are increased in other neurodegenerative disease such as ALS or Parkinson's disease, but are decreased in AD (Appel and Sengun 2003). As to organ specific CNS antigens, a decreased incidence of autoantibodies against gm1 gangiliosides in CSF was observed (Richartz et al. 2004]). Moreoever, the natural antibodies against amyloid protein supporting the degradation of cerebral ß-amyloid, are decreased in AD patients (Du et al. 2001; Weksler et al. 2002).

Taken together, investigations of autoantibodies remained contradictory. The results did not sustain the neuroautoimmune model (Aisen and Davies 1994; Singh 1997) suggesting that neurodegeneration in AD is a consequence of classical autoimmune processes.

Rather, recent findings point to a decrease instead of an increase of antibody concentrations (Richartz et al. 2004).

With the development of more sophisticated techniques, the investigation of cytokines as essential immune mediators advanced, and studies on cytokine alterations of cytokines seemed more promising.

As to their origin, it seemed reasonable to postulate a link between the cytokine profile in the blood stream and that in the brain, because there is an active and highly regulated communication between the brain and the immune system (Huberman et al. 1994). On this background, several studies on inflammatory markers in serum and CSF in AD patients have been carried out, in attempt to find a premortem diagnostic marker for AD. First, it seemed consequent that the local inflammatory processes would be associated with systemic inflammatory signs. However, data remained inconsistent and, hitherto, do not allow drawing definite conclusions. Guided by cerebral findings, numerous studies focused on the peripheral secretion of proinflammatory cytokines. In CSF, increased levels of proinflammatory cytokines (Bagli et al. 2003; Blum-Degen et al. 1995), unchanged levels (Lanzrein et al. 1998; März et al. 1997; Tarkowski et al. 1999) and decreased levels (Singh 1994; Yamada et al. 1995) have been found in AD. Of similar inconsistence are the findings in serum: Some working groups report elevated levels of proinflammatory cytokines (Kalman

et al. 1997; Licastro et al. 2000; Lombardi et al. 1999; Singh and Ghutikonda 1997), other do not see any changes (Androsova et al. 1995; Esumi et al. 1991; Lanzrein et al. 1998), while several find a decrease of proinflammatory cytokine secretion (Cacabelos et al. 1994; De Luigi et al. 2001; Paganelli et al. 2002; Sala et al. 2004). These discrepancies have mostly been attributed to technically different approaches and to different criteria to choose patient groups as well as control groups. Moreover, most of the studies report very low cytokines levels nearby their detection limit, so that statistical evaluation is restricted. However, within the confusing variety of systemic findings it is becoming increasingly substantiated that AD patients exhibit systemic immunological alterations, which do not just reflect the inflammatory processes in the brain. It has been stated that the neuroinflammatory events found in the brain and CSF of AD patients seem to be limited to the CNS without direct association of a peripheral inflammation (Blum-Degen et al. 1995).

Own studies were carried out on the hypothesis that AD patients display systemic immunological alterations in terms of a dysregulation or impairment of the immune response, which do not only reflect an epiphenomenon, but may causally be related to the Alzheimer's pathology (Richartz et al. 2005). On the assumption that various immune functions, not only of the proinflammatory response, are hampered in AD, we investigated the cytokine secretion of TH 1 cells, TH 2 cells, as well as of the macrophage/moncyte system. In a preliminary study, we measured the concentrations of the proinflammatory cytokines IL-1ß, IL-2, IL-6, and TNF- α, as well as of the soluble receptors sIL-2r, sIL-6r, and sTNF-αr in cerebrospinal fluid (CSF) and in serum of Alzheimer patients and controls. With respect to the low concentration values, we then stimulated whole blood cell cultures with mitogens, leading to higher cytokine levels. After mitogenous stimulation, we measured the increase of cytokine levels above basal levels of the proinflammatory cytokines IL-6, IL-12, IFN-y and TNF-α, and of the antiinflammatory cytokines IL-5 and IL-13.

2 Subjects and Methods

Recruitment of AD patients was done at the University Clinic for Psychiatry Tuebingen, Germany. The diagnosis of probable AD was performed according to the NINCDS-ADRDA criteria (McKhann et al. 1984). Control subjects for CSF and serum investigations were chosen from the Department of Neurology, Goettingen, Germany. Lumbar punction was carried out either in patients with questionable disc prolapse, who underwent radiological examination with contrast medium, or in patients suspected of having an inflammatory or other CNS disease. Their CSF status was normal as regards cell count, albumin and IgG, as were all measured serum parameters. Any organic CNS disease was excluded in all of these persons. For cell cultures, control blood was gained by healthy aged persons, who were recruited through advertisement in the local press.

A comprehensive somatic, psychiatric, and socio-demographic history was taken of all persons. All subjects underwent thorough psychiatric and neurological examination including EEG and neuroimaging (CT or NMR). Cognitive decline was measured by the Mini Mental State Test (MMST, Folstein et al. 1974). Total blood count and blood chemistry including C reactive protein, thyroid function, vitamin B12, Folic acid, Borrelia and Lues serology was evaluated. Patients with a psychiatric, neurological, inflammatory or infectious disease or with a history of immunological or malignant disease were excluded, as well as persons with abnormal white blood cell count, C reactive protein or signs of malnutrition. Further exclusion criteria were the intake of immunologically relevant or psychotropic drugs and a positive family history for dementia. All control subjects underwent the same clinical examinations including MMST and laboratory tests as the AD patients. The same exclusion criteria were applied. MMST of controls had to be normal.

In vivo concentrations of cytokines and soluble receptors in CSF and serum were determined in twenty patients with probable AD (16 female and 4 male, 60–88 years, median 72 years). The MMST score was in the range of 10–23, with a median of 16. As controls, we investigated CSF and serum samples from 21 subjects (7 female, 14 male, 59–82 years, median 68 years). For studying cytokine production in stimulated blood cell cultures, further 27 patients, 18 of them females, 9 males, with probable AD and 23 healthy aged volunteers, 16 females and 7 males, were included. The median age of the Alzheimer patients was 70 years (63–84 years), of the control persons 68 years (59–77 years). The MMSE score ranged between 11 and 21 in the patient group (Median: 17.3). Mean of Alzheimer disease duration was 2.5 years (1.5–3.4 years). The groups for native and stimulated cytokine investigations were comparable with respect to age and disease duration.

The investigation was carried out in accordance with the Declaration of Helsinki. Written informed consent was given from all subjects or their relatives following full explanation of the procedure. The study was carried out after approval by the local ethics committee.

Samples were collected at routine venipuncture between 8:00 and 9:00 am in order to take in account the circadian rhythm. For in vivo cytokine measurement, blood samples were centrifuged and the serum frozen at–20° C until analysis. CSF was obtained by lumbar punction, centrifuged and frozen at–20° C until analysis. For blood cell stimulation, whole blood samples were cultured following the Lubeck protocol (Kirchner et al. 1982). Peripheral blood cells were stimulated with LPS and PHA, for 48 and 96 h, respectively. After centrifugation supernatants were stored at –80° C until measurement. Cytokine concentrations were determined using commercially available ELISA kits (IL-1ß, IL-6, TNF-α, IFN-γ, sIL-2r:Milenia, Bad Nauheim, Germany; IL-5, IL-12, IL-13, sIL-6r, sTNF-αr:R&D Systems, Wiesbaden, Germany). Based on preliminary experiments, for each cytokine the time of stimulation was chosen according to the time of maximal induction. IL-5, IL-6, IL-13, and TNF-α were measured after 48 h of stimulation, IL-12 after 72 h, IFN-γ after 96 h of stimulation.

For statistical analysis, the differences between the patients and control groups were analyzed by Wilcoxon rank sum test and χ^2 test. The Bonferroni adjustment for multiple comparisons was applied.

3 Results

3.1 In Vivo Concentrations of Cytokines and Soluble Receptors in CSF and Serum

The data of this study are compiled in Table 1. The concentration of IL-2 in CSF as well as serum levels of IL-1ß, IL-2 and TNF-α were too low to reach detection limit. Regarding the other values, we found a decrease of all parameters in CSF and serum of the AD patients compared with the control group. Considering a p-value of less than 0.005 (n=10), Bonferroni adjustment showed a statistically significant decrease of TNF-α in CSF (p <0.0001) and of IL-6 in serum (p <0.0012) of the AD patients. There was no effect of gender (Kendall tau b correlation) and age (Pearson correlation). The diminished levels were not correlated with disease duration (MMST values) or severity.

3.2 Production of Cytokines in Stimulated Blood Cell Cultures (Figs. 1, 2)

We determined the ability of blood cells to produce the proinflammatory cytokines IL-6, IL-12, TNF-α and IFN-γ, and the T-helper (TH)-2-cell derived antiinflammatory cytokines IL-5 and IL-13. As illustrated in figs. 1, 2, the AD group shows reduced levels of all cytokines after mitogen-induced whole blood stimulation in comparison with the control group. On account of Bonferroni adjustment, a p value

Table 1 Cytokine concentrations in CSF and serum (pg/ml): mean and standard error of the mean (S.E.M.) (in parentheses); (*) = p < 0,005 (Bonferroni adjustment); "-": levels under detection limit

	CSF		Serum	
	AD	Controls	AD	Controls
IL-1ß	19,6 (2,0)	23,3 (2,1)	-	-
IL-2	-	-	-	-
sIL-2r	47,6 (1,85)	55,6 (2,97)	421 (35,57)	447 (37,55)
IL-6	4,6 (0,48)	10,6 (4,44)	4,7 (2,4) (*)	16,1 (3,04)
sIL-6r	575 (38,70)	767 (22,43)	21,03 (1,89)	24,08 (1,36)
TNF- α	14,0 (0,37) (*)	19,3 (0,43)	-	-
sTNF-αr	681 (33,78)	667 (34,04)	1,527 (1,88)	1,94 (0,27)

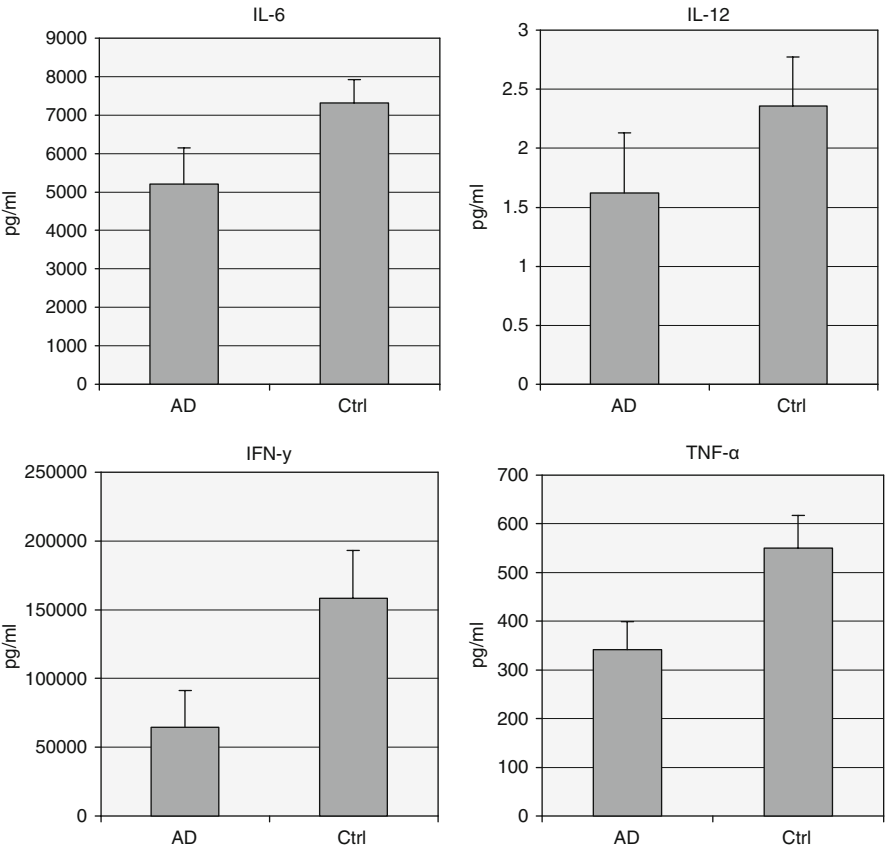

Fig. 1 Release of proinflammatory cytokines (in pg/ml, with SEM) in mitogen-stimulated whole-blood cell cultures from AD-patients (AD) and controls (Ctrl)

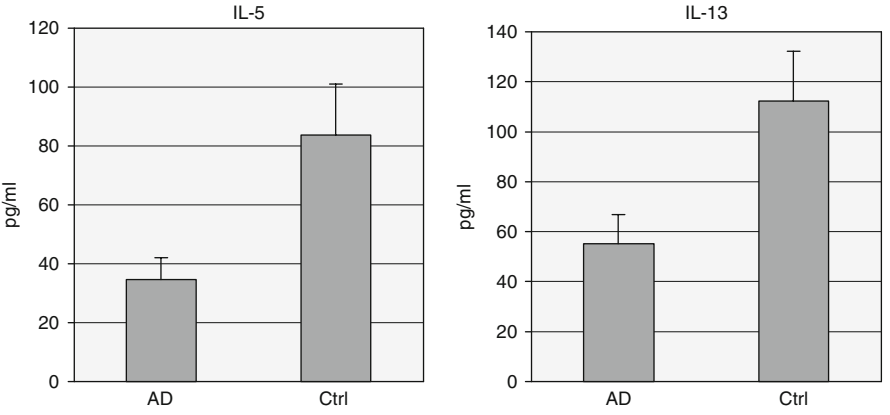

Fig. 2 Release of antiinflammatory cytokines (in pg/ml, with SEM) by mitogen-stimulated whole-blood cell cultures from AD-patients (AD) and controls (Ctrl)

of less than 0,008 (n=6) was considered statistically significant. Thus, a high significance was shown for the decrease of IL-6 ($p<0.001$), IFN-γ ($p<0.0002$), TNF-α ($p<0.0005$) and of IL-5 ($p<0.001$). IL-12 was decreased with $p<0.019$, IL-13 with $p<0.023$. The results remained significant also after stepwise regression control to exclude the possible influence of age and sex. No correlation was found between the cytokine levels and duration of disease or severity of disease, respectively.

4 Discussion

The role of the immune system in the pathogenesis of AD has been widely discussed. Since AD is no longer regarded as a single unified condition but as a complex syndrome, it has been postulated that the presence of different clinical subgroups may imply a differential involvement of the immune system (Huberman et al. 1994; Licastro et al. 2000).

4.1 Cytokine Measurement in AD

The literature on peripheral cytokine secretion in AD is various, and findings remain inconsistent and intricate to interprete. Obvious methodological differences among studies, including inclusion criteria and differences in the techniques used to measure cytokines contribute to the great variability of data. Sample sizes show considerable differences, and the patient groups differ with respect to stage of dementia, further pathological conditions and drug intake. Moreover, varying cytokine levels may also be due to genetic polymorphisms (Bagli et al. 2003). Therefore, the measurement of a single cytokine does not allow any conclusions on disease dependent effects. Rather, an overlapping set of cytokines as presented in this study may give more information. Most importantly, cytokine production is highly dependent on the health status. Previously reported higher levels of proinflammatory cytokines in aged persons as well as in AD might reflect an underlying but undiagnosed disease state (Beharka et al. 2001). On this background, in our study we excluded each person with the slightest sign of infection or other medical disease, because any comorbidity could influence the cytokine production. Moreover, since treatment with acetylcholinesterase inhibitors may modulate cytokine expression (Reale et al. 2004) patients only were included before starting antidementive therapy. The measurement of cytokine secretion was done using stimulated whole blood cell cultures. Whole blood cultures resemble more closely the in vivo situation since manipulation, prestimulation, and possible selection of PBMC are minimized, and the role of plasma factors is included.

We observed diminished levels of proinflammatory cytokines in CSF and serum and of the soluble receptors in the AD group compared with healthy, aged controls. In summary however, these in vivo concentrations have been shown to be very low.

Critical parameters influencing cytokine levels in CSF are, e.g., the relatively large volume and the dynamics of the CSF system, the brain CSF barrier as well as the distance of the liquor system from the relevant brain regions (März et al. 1994). Similarly, some native cytokine concentrations in serum were near or under the detection limit. In contrast to our results, other investigators were able to found measurable cytokine levels. This discrepancy could be explained by undiagnosed comorbidity or intake of drugs leading to altered cytokine secretion. More important may be technical differences, particularly concerning origin, structure and sensitivity of the antibodies applied in the different ELISA kits.

Findings in stimulated blood cell cultures are much more expressive, since cytokine levels are markedly higher, and differences between groups are depicted more clearly. Moreover, the relative increase of cytokine levels upon stimulation reflects the functional responsiveness of the particular immune cells on inflammatory stimuli. In our study, the increase of all measured cytokines, i.e. IL-5, IL-6, IL-12, IL-13, TNF-α and IFN-γ in whole-blood cell cultures stimulated with mitogens, is significantly lower in AD patients than the increase of cytokine levels in the control group. The finding of an unidirectional decrease of all measured cytokines points to a general dysfunction of the cellular immune response to stimulating agents. The main source of IL-6, IL-12, TNF-α and IFN-γ is the monocyte/macrophage system. Moreover, IFN-y, and to a lower degree TNF-α, are also expressed by TH-1 cells. TH-1cells play a central role in the activation of the monocyte system. Additionally, they induce B-cells to produce opsonizing antibodies. Opsonizing, again, promotes phagocytosis. Thus, a diminished production of these cytokines may be associated with an impaired phagocytic activity. As phagocytosis is essential for the removal of foreign bodies, debris and dysfunctional proteins, impairment can lead to accumulation also of amyloid proteins as is the case in a number of local and systemic amyloid diseases (Linke 1996). In contrast, IL-5 and IL-13 derive from TH-2 cells and act as antiinflammatory immune mediators. Interestingly, their expression has been found to be significantly decreased as well. Taken together, we see a generally blunted secretory response of immune cells on activating stimuli in AD. This observation is in contrast to the protective effect of antiinflammatory drugs when taken for long term before the onset of AD, as seen in several epidemiological studies. However, a therapeutic effect of antiinflammatory substances is up to now not proved in prospective clinical studies. Moreover, the histopathological evidence of proinflammatory molecules in the diseased brain is not necessarily in contrast to the assumption of an underlying general immune depression. A decline of phagocytic activity as one of the beneficial effects of the immune response may constitute an early event in the pathogenetic chain. However, the local overproduction of inflammatory markers have been attributed to a secondary reaction to the accumulating amyloid burden (Mc Geer and McGeer 2003) obviously overtaxing the phagocytic capacities of the AD brain. Finally, the mechanism of the antiinflammatory drug effect in AD is not yet clarified. Possibly, they do not act via inhibition of the prostaglandinsynthesis, but through reduction of the amyloid burden (Cirrito and Holtzmann 2003).

4.2 Consistent Findings Indicating an Immune Dysfunction in AD

Several studies point to an at least partial impairment of the immune system in AD. A decreased production of TNF-α in mild stages of AD was interpreted as a sign of defective immune functions (Huberman et al. 1994). Phytohemagglutinin (PHA)—stimulated proliferation and IL-2 production of nonadherent monocytes in AD patients has been shown to be significantly reduced (Fujiwara 1996). The lack of proliferative responsiveness to APP peptides in AD led to the assumption of a "T-cell anergy" in AD (Trieb et al. 1996). A generally decreased in vitro T-cell-activation to a number of stimuli in AD has been reported, and an increase of acute reactants is interpreted as a compensatory reaction to in vivo functional alterations of leukocytes (Dickson et al. 1996). Other studies have shown imbalances of cellular immunity and immunoregulatory T-cells and a reduced T-cell response to various antigenic determinants suggesting a defect of the T-cell mediated immunity in AD (Giubilei et al. 2003; Streit 2001). Accordingly, a decrease of proliferation activity of AD lymphocytes has been reported, subsequently resulting in the impairment of immune functions in AD (Zhang et al. 2003). These functional defects have been attributed to oxidative damage of DNA in lymphocytes from AD patients (Mecocci et al. 1998) and an altered calcium response of peripheral T-lymphocytes in AD (Sulger et al. 1999). Most interestingly, an accelerated telomere shortening in lymphocytes has been found as an underlying cause of the impaired lymphocyte function in AD (Panossian et al. 2003; Zhang et al. 2003).

4.3 Putative Causal Role of Immune Dysfunction in AD

The question of a pathogenetic role of the immune dysfunction in AD is matter of ongoing discussion. One hypothesis suggests that a peripheral immune impairment is an epiphenomenon, secondary to the central immune activation seen in AD. Via the hypothalamic pituitary axis the cerebral inflammation may lead to an increased production of cortisol, resulting in a peripheral immunodepression (Woiciechowsky et al. 1999). Indeed, a mild hypercortisolemia has been shown in AD patients (Hartmann et al. 1997).

On the other hand, a causal role of an underlying general impairment of the immune response in AD seems conceivable with respect to three major points of view:

4.3.1 Microglial Dysfunction in AD

The role of immunological and inflammatory processes in the pathogenesis of AD is widely understood in terms of the "bystander damage hypothesis" (Streit 2002).

Accordingly, the neurodegeneration in AD is caused through bystander damage from autoaggressive microglial cells that produce neurotoxins in response to continue A ß exposure (Akiyama et al. 2000; McGeer and McGeer 2001). However, the primary function of microglia is to support neuronal survival and regenerative processes including phagocytosis (Rogers et al. 2002; Streit 2002). The role of microglia in the degradation and clearance of cell debris as well as of amyloid proteins is meanwhile well established (Popovic et al. 1998; Streit 2001). Microglia derives from the same stem cells as monocytes and have been shown to undergo similar functional impairment in AD as assumed for the peripheral monocytes of AD patients (Streit 2001; Fiala et al. 2002). Histopathological studies on AD microglia showd altered morphology indicating a functional impairment (De Witt et al. 1998; Sasaki et al. 1997). The long-term presence of activated microglia around ß-amyloid plaques has been referred their inability of phagocytosing and clearing senile plaque cores (Apelt et al. 2001). Microglial dysfunction may become manifest in a number of ways, including a decreased ability to produce neurotrophic factors, a decreased phagocytic capacity, as well as increased neurotoxicity (Streit 2002). These alterations may be of pathogenetic relevance in AD. It has been shown that deficient phagocytosis promotes inflammation and can lead to immune-mediated tissue degeneration (Wyss-Coray and Mucke 2002). Presumably, chronic struggle of microglia to remove Aß-containing plaque material promotes inflammatory processes in AD (Lue and Walker 2002). These changes are assumed to be age-related, but are pronounced in AD.

Taken together, findings of a systemic attenuation of cellular immune response may be related to the cerebral pathology in AD in terms of insufficient phagocytosis of amyloid proteins and resulting neurotoxic effects.

4.3.2 Decrease of Amyloid Burden Through Immunstimulation

The assumption of a causal significance of an immunological impairment in AD is even more intriguing in the light of the studies on immunization with ß -amyloid. Vaccination of transgenic mice with ß-amyloid leads to an enhanced removal of amyloid deposits in the brain (Schenk et al. 1999) by promoting microglial phagocytosis. While the exact mechanisms are still point of discussion, also peripheral mechanisms have been considered (Lemere et al. 2003). Peripheral immune cells have been shown to invade the brain of adult mice as well as AD brain (Eglitis and Mezey 1997, Fiala 2002). Possibly, immunization leads to a peripheral immune response, which via penetration of T-cells and macrophages into the brain will enhance phagocytosis of local Abeta. Furthermore, immunstimulation with LPS results in reduction of ß-amyloid plaques in APP PS1 transgenic mice what has been shown for direct intrahippocampal injection (DiCarlo et al. 2001) as well as for systemic administration of LPS (Quinn et al. 2003).

In view of a putatively underlying immune deficit and impaired phagocytotic activity in AD, the effect of immunization or immunstimulation leading to a decrease of the cerebral amyloid burden seems consistent and conceivable.

4.3.3 Role of Aging

Finally, there seems to be an obvious association between the immune alterations seen in AD and aging processes. Immune-aging phenomena constitute a major risk factor for AD (Blasko and Grubeck-Loebenstein 2003; Gasiorowski and Leszek 1997). The role of aging in AD development is conspicuous since epidemiological studies identified advanced age as the only consistent risk factor for AD. The age-dependent decrease of immune functions does not only involve the adaptive immunity (Blasko and Grubeck-Loebenstein 2003), but the innate immune system as well. T-cell derived cytokine production decreases with aging (Esumi et al. 1992; Gillis et al. 1981), and in vitro lymphocyte responsiveness to activating agents (e.g., lectins) has been shown to be reduced in elderly humans in several studies (DiCarlo et al. 2001). Macrophages, as well, underlie age-associated functional alterations (Lloberas and Celada 2002).

Obviously, the immunological alterations in AD patients are more pronounced than the age-related changes in healthy persons. The T-cell observations in AD patients are characteristic of T-cells that reach a state of high replicative senescence after multiple rounds of antigen-induced cell-division (Effros 1998; Panossian et al. 2003).

Moreover, AD patients show, in comparison with healthy aged people, increased mitochondrial DNA mutations and genomic DNA damage which can lead to dysfunction and decline of PBMC (De la Monte et al. 2000).

On this background, the observations of a blunted T-cell–response in AD patients finally could be understood as sequel of a premature immunosenescence, presumably being one important factor within the multifactorial etiopathogenesis of AD. This assumption is substantiated by the parallels between AD patients and patients with Down syndrome (DS). DS patients suffer from progerie and are of high risk to develop AD. Interestingly, DS patients show similar signs of advanced immunological senescence as seen in AD, such as telomere shortening (Park et al. 2000; Zhang et al. 2003) and altered intracellular calcium responses of T-cells, which might negatively influence the T-cell help required to generate an effective antibody response to A ß (Grossmann et al. 1993).

This study is limited due to the small amount of data and the heterogeneity of patients in terms of age, disease duration and severity. However, the present data support alternative views to the hypothesis of a mere inflammation-mediated pathogenesis, particularly since trials with antiinflammatory agents have not yet shown a clear benefit in preventing or delaying disease onset. Our hypothesis of a premature immunosenescence as a pathogenetically relevant factor in AD is in line with a "gerocentered" view rather than a just "amyloidocentered" approach in understanding the etiology of AD (Joseph et al. 2001). Conclusively, the development of therapeutic strategies which stimulate the general immune responsiveness seems to be a promising challenge for future research.

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Frailty

Inflammatory Markers and Frailty

Sean X. Leng and Linda P. Fried

Contents

Abstract: As the aging population increases rapidly worldwide, caring for frail older adults has become the mandate of modern medicine. As such, frailty has been increasingly recognized as an important geriatric syndrome. This is further supported by the recent development of an operational definition, validation of a set of criteria, and evidence for its syndromic nature. Frailty is characterized by decreased functional and physiologic reserve, increased vulnerability to stressors, as well as high risk for serious adverse health outcomes including disability, dependency, and mortality. Although the pathogenesis of this syndrome is far from being elucidated, frail older adults demonstrate dysregulations in multiple physiologic systems. As discussed elsewhere in this handbook, low grade, chronic systemic inflammation manifested in older adults, so-called "inflamm-aging," is an important feature of immunosenescence. Activation of the inflammation system marked by elevated levels of inflammatory markers, above and beyond age-related increases, is considered the most prominent pathophysiological feature of frailty. This chapter provides an overview of the syndrome of frailty and its relationship with several molecular and cellular inflammatory markers, including interleukin-6 (IL-6), C-reactive protein (CRP), and white blood cell (WBC) and its specific subpopulations. It also discusses the potential role of chronic systemic inflammation, directly and/or through other intermediary systems, in the pathogenesis of frailty.

Keywords: Frailty • Inflammatory Markers • IL-6 • WBC • CCR5+ T Cells

S. X. Leng (✉) · L. P. Fried
Division of Geriatric Medicine and
Gerontology and Center on Aging and Health
Johns Hopkins University School of Medicine
John R. Burton Pavilion, 5505 Hopkins Bayview Circle
Baltimore, Maryland 21224, USA
Tel.: 410-550-2494; Fax: 410-550-2513
E-mail: sleng1@jhmi.edu

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1 The Geriatric Syndrome of Frailty

The evaluation and treatment of frail older patients constitute a cornerstone of the care for older adults. Until recently, frailty has been a term that is used more frequently than it is defined. Geriatricians have long been aware of a syndrome of multiple coexisting conditions, weakness, immobility, and poor tolerance to physiologic or psychological stressors. Due to lack of diagnostic criteria, geriatricians say, "I know it when I see it. But, what I see may not be the same as everyone else sees. " Given the ever growing of older adult population, particularly the rapid expansion of the segment aged 85 years and older (the "oldest old"), searching for a standardized definition of frailty and understanding its pathophysiologic basis has become paramount.

Recent work led by Fried and colleagues suggests that frailty as a syndrome in old age and a state of decreased physiologic reserve and high vulnerability for subsequent morbidity and mortality [1–4]. Frailty has also been described as a syndrome with a loss of complexity in resting dynamics involving multiple organ systems, manifested by maladaptive responses to stressors, leading to a vicious cycle toward functional decline and other adverse clinical outcomes [5, 6]. The phenotypic characteristics of frail older adults is now recognized to be a syndrome consisting of three or more of the following: weakness, low physical activity, slowed motor performance, exhaustion, and weight loss [1, 6]. The presence of three or more of these characteristics is independently predictive of a number of serious adverse health outcomes, including acute illness, falls, hospitalization, disability, dependency, and early mortality, adjusting for comorbidities [1]. The estimated prevalence of this syndrome is 7–10% among community-dwelling men and women age 65 and older, and up to one-third of those aged 80 years and older [1, 6]. The phenotypic characteristics described above, of which three appear to be the syndromic critical mass, have been validated by many large cohort studies and in various clinical and cultural settings [1, 7–11]. This frailty index has also been favorably evaluated and compared with other proposed frailty criteria [12]. A recent American Geriatric Society and National Institute on Aging-sponsored national conference in the US on the research agenda on frailty has further utilized Fried's index as the preliminary criteria for frailty [3, 4].

Based on the above validated and now widely utilized frailty criteria, the manifestations of frailty, as a clinical syndrome, encompass a constellation of symptoms including weakness, fatigue, inactivity, unintentional weight loss, and decreased food intake. Signs of frailty that are often cited include sarcopenia (loss of muscle mass), balance and gait abnormalities, deconditioning and decreased bone mass (Fig. 1). Weakness (measured by muscle strength or power) and slowed motor performance (measured by walking speed) appear to be the cardinal signs of the frailty syndrome. Consistent with its definition as a syndrome, the symptoms and signs of frailty may vary across this constellation of possible manifestations, with multiple components present, but not always the same ones from patient to patient.

Frailty is recognized as a distinct clinical entity distinguished from comorbidity and disability, two other prevalent conditions in older adults [1, 6]. All three

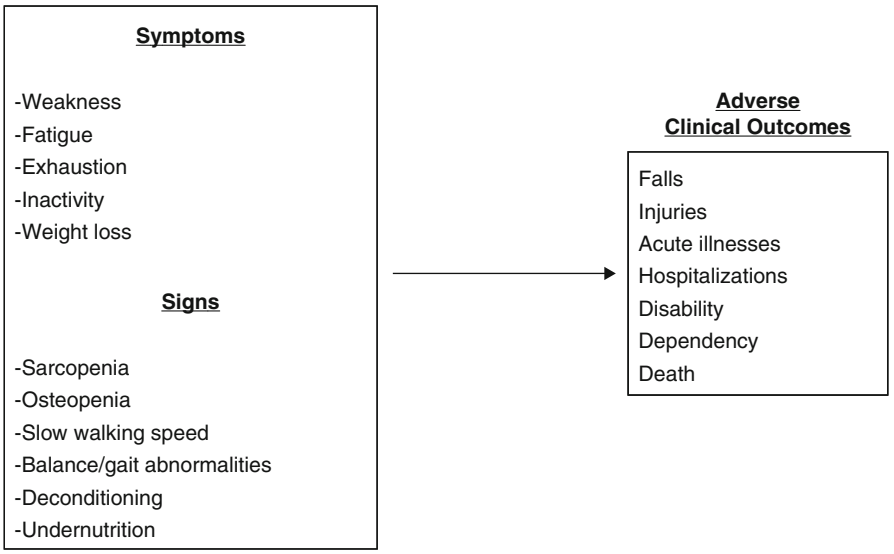

Fig. 1 Clinical manifestations and consequences of the geriatric syndrome of frailty

conditions are predictive, at various degrees, of adverse clinical outcomes; therefore, have significant overlap (Fig. 2). However, the main features of frailty (including decreased functional reserve, impairment in multiple physiological systems, and reduced ability to regain physiological homeostasis after a stressful and destabilizing event) make the distinction of frailty from disability or comorbility relatively easy. Disability suggests chronic limitations or dependency in mobility and/or activities of daily living (ADLs: eating, bathing, dressing, toileting, and ambulating) or instrumental activities of daily living (IADLs: shopping, housekeeping, cooking, driving, taking medications, and handling finance). While many (but not all) frail individuals are disabled, not all disabled persons are frail. For example, older patients who suffer severe disability secondary to a major cerebral vascular accident or stroke may maintain relatively intact function in other physiological systems and

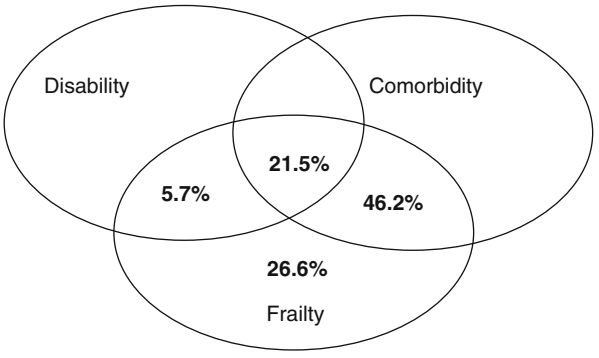

Fig. 2 Venn diagram demonstrating the overlap of frailty with disability and comorbidity. Adapted from Fried LP, et al (2001) Frailty in older adults: evidence for a phenotype. J Gerontol A Biol Sci Med Sci 56:M146–156

thus, are not frail. As time goes by, these individuals may develop frailty if they are not recovered from their disability. Therefore, disability is likely an outcome of frailty or a contributor to frailty. Comorbidity indicates the presence of multiple chronic diseases. Not surprisingly, comorbidity is associated with increased risk of adverse outcomes, as evidenced by higher short-term and long-term mortality and significantly increased physical disability compared with those without diseases. However, the mere presence of two or more diseases in itself, even if in relatively severe forms, may not identify the vulnerable group of older patients or those who are frail. Again, if these comorbid conditions are not adequately treated and/or more diseases are accumulated, these patients may develop frailty [6].

The etiology for the syndrome of frailty is current unknown. Old age is clearly a significant risk factor for frailty, as the prevalence of frailty increases with age [6]. Older adults can develop frailty in the absence of any clinically evident diseases (primary frailty). Frailty can also develop from multiple coexisting diseases, often chronic conditions but can be triggered by acute episodes of existing conditions or acute new diseases, as a common pathway (secondary frailty) to disability, dependency, and death [2]. Emerging evidence suggests that chronic systemic inflammation is the most prominent pathophysiological feature of frailty and may play a critical role in the pathogenesis of this syndrome.

2 Molecular Inflammatory Markers and Frailty

As an important feature of the immunosenescence, aging is characterized by a low grade, chronic systemic inflammatory state, so-called “inflamm-aging” [13]. This inflammatory phenotype is marked by elevated levels of molecular and cellular inflammatory markers and is associated with increased morbidity and mortality in older adults [14]. Inflammatory molecules with age-related increase in their levels include proinflammatory cytokines and their receptors, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), Interluekin-1 receptor antagonist (IL-1Ra), and soluble TNF receptors, diverse chemokines, such as CXCL10 (also termed interferon-gamma induced protein 10, or IP-10), regulated upon activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein-1 alpha (MIP-1a), monocyte chemoattactant protein-1 (MCP-1), and IL-8, as well as C-reactive protein (CRP) [15–19]. The following will focus on IL-6 and CRP for which recent studies have demonstrated elevated levels in frail older adults, above and beyond age-related changes.

2.1 IL-6 and Frailty

IL-6 is a proinflammatory cytokine with elevated circulating levels in older adults [20, 21]. Age-related increases in IL-6 levels are associated with several pathophysiologic processes, including atherosclerosis, osteoporosis, and sarcopenia, and with functional

decline, disability, and all-cause mortality in older adults [21–26]. In addition, elevated IL-6 levels are associated with decreased muscle mass and strength even in well-functioning older men and women [27, 28]. In a longitudinal study, Ferrucci and colleagues reported that elevated IL-6 levels at baseline predict a significantly higher risk for the development of physical disability and a steeper decline in muscle strength and walking performance during a follow-up period of 3.5 years in older women living in the community [29]. This study and others have shown that chronic systemic inflammation marked by elevated IL-6 levels is associated with decreased muscle strength and power and slowed walking speed, two central components of the frailty syndrome. Direct evidence supporting the relationship of this molecular inflammatory marker with frailty came first from a pilot study in which community-dwelling frail older adults had significantly higher IL-6 levels than nonfrail controls with similar age [30]. A subsequent age- race and sex matched pair study has further demonstrated that frail older adults living in the community had significantly higher IL-6 production by the peripheral blood mononuclear cells (PBMCs), upon stimulation with lipopolysaccharide (LPS), compared to the matched nonfrail controls [31]. Furthermore, two recent studies in large cohorts of community-dwelling older women have demonstrated that elevated IL-6 levels are independently associated with the syndrome of frailty [32, 33]. These clinical, laboratory, and population studies have provided strong evidence for the contributory role of this important proinflammatory cytokine to frailty in older adults.

2.2 CRP and Frailty

CRP, discovered in 1930 as an acute phase reactant, is a classic circulating molecular marker of systemic inflammation [34]. Elevated CRP levels are associated with many late-life chronic conditions, including Alzheimer's disease, cardiovascular diseases, macular degeneration, and functional decline, disability, as well as all-cause mortality in older adults [24, 26, 35]. Clinically, CRP has now been integrated as part of the routinely measured panel of cardiovascular disease risk factors. Two large cohort studies have demonstrated the direct association of this molecular inflammatory marker with frailty. In the Cardiovascular Health Study (CHS), Walston and colleagues have shown that significant association of elevated CRP levels with frailty after excluding cardiovascular disease and diabetes and adjusting for basic demographic characteristics [36]. Data from the Longitudinal Aging Study Amsterdam (LASA) have further confirmed these findings [37]. These studies suggest that CRP, along with IL-6, is an important circulating molecular inflammatory marker for the syndrome of frailty.

3 Cellular Inflammatory Markers and Frailty

White blood cell (WBC) and its subpopulations are circulating immune cells and an important cellular component of the inflammation system. Total WBC count is a stable, well-standardized, widely available, and inexpensive cellular inflammatory

marker. Clinically, increase in total WBC counts (above the normal range) is recognized as a marker of systemic inflammation, primarily secondary to acute bacterial infections. Numerous studies, particularly several recent large cohort studies in older adults, have demonstrated that elevated WBC count is associated with cardiovascular and cerebrovascular events, cardiovascular and cancer mortality, as well as all-cause mortality [38–40]. The predictive value of elevated baseline total WBC counts for all-cause mortality of community-dwelling older women remained after excluding those with high WBC counts above normal range and hematological malignancies[38]. The relationship of this well-recognized cellular inflammatory marker with frailty has been recently evaluated, demonstrating that total WBC counts had independent association with frailty in older women living in the community [32]. As shown in Fig. 3, results from this study showed significant trend of increase in risks for frailty from participants in the bottom tertiles of both total WBC counts and IL-6 levels (reference group) to those in the mid tertiles and those in the top tertiles, suggesting a potential synergistic interaction between these two common cellular and molecular inflammatory markers in their associations with frailty [32]. This was supported in part by the study cited above in which PBMCs, isolated WBC subpopulations, from frail older adults had significantly higher LPS-induced IL-6 production than that from matched nonfrail controls [31]. Another study has shown direct associations of circulating IL-6 levels and total WBC and differential counts in the same cohort of older women living in the community [41].

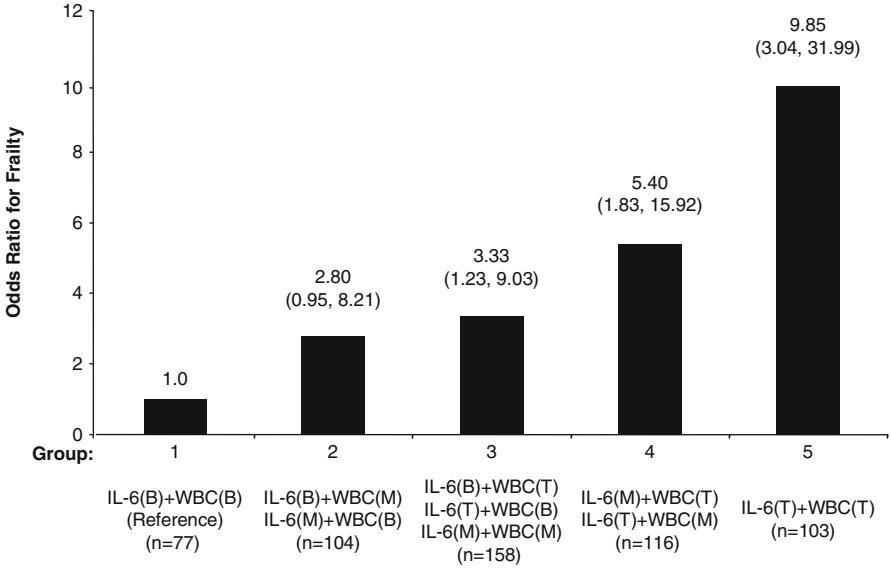

Fig. 3 Odds ratios for frailty in each of the five groups of participants from the Women's Health and Aging Studies I:Participants were grouped based on their tertiles of IL-6 and WBC: (B)–bottom tertile; (M)–mid tertile and (T)–top tertile. The numbers of participants in each group are indicated in parentheses

In terms of WBC subpopulations, ongoing studies have shown that counts of neutrophils and monocytes are significantly associated with frailty (Leng S, unpublished data). Lymphocytes and their overall effects are difficult to assess as they are consisted of heterogeneous subsets with diverse immune regulatory functions (Th1 and Th2 proinflammatory phenotypes vs. T regulatory or suppressor phenotypes, etc.). In addition, drastic and ongoing remodeling, particularly in the T-cell compartment, occurs during aging. Although no consistent association between total lymphocyte counts and frailty has been demonstrated, specific T-cell subsets have been reported to have significant associations with frailty. For instance, it is well documented in the immunosenescence literature that CD8+ and CD8+CD28- subsets of T-cells experience most consistent expansion during aging. The *post hoc* analysis in a recent study evaluating the relationships between T-cell subsets and mortality suggests that frailty is associated with increased CD8+ and CD8+CD28- T-cells in older women [42]. Chemokine CC receptor-5 (CCR5) is a well-known coreceptor for macrophage (M) and dual (T-cell and M)—tropic human immunodeficiency virus type-1 (HIV-1) infection [43]. CCR5+ T-cells have a proinflammatory and type-1 phenotype [44, 45, 47] and contribute significantly to several inflammatory conditions [44–48]. In a study of 13 frail and age- race, sex, matched nonfrail older adults living in the community (mean age 84 years), frail participants had significantly higher percentage of CCR5+ T-cells in the total T-cell pool than matched nonfrail controls (Fig. 4) [49]. These studies suggest that frailty is associated with increased frequencies of CD8+, CD8+CD28-, and CCR5+ T-cell subsets above and beyond age-related T-cell remodeling. However, whether these T-cell subsets possess proinflammatory function in frail older adults as well as their role in the development of frailty remain to be determined.

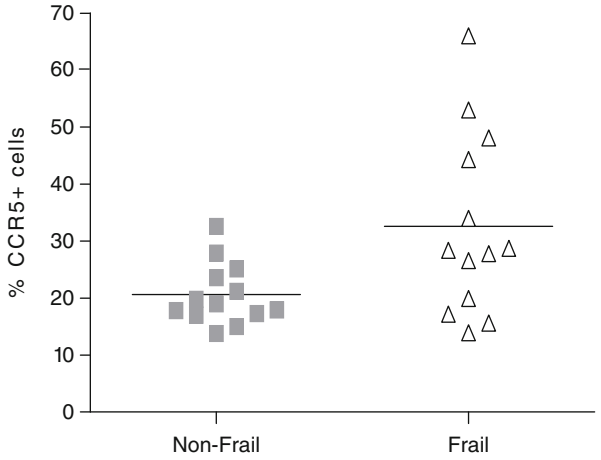

Fig. 4 Percentages of CCR5+ T-cells in 13 frail and matched nonfrail older adults: Each symbol represents one participant. Horizontal lines denote the means

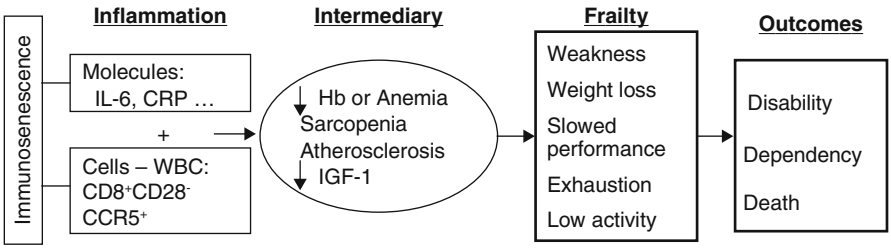

Fig. 5 Hypothetical inflammation model pathway to frailty in older adults

4 Role of Inflammation in Frailty

As discussed above, the associations between frailty and common molecular and cellular inflammatory markers are well documented. The critical question is whether chronic systemic inflammation plays a role in the pathogenesis of frailty. As noted earlier, individual inflammatory molecules, such as IL-6, can directly contribute to frailty or its central components (such as decreased muscle strength/power and slowed motor performance). In addition, frailty involves multiple physiologic organ systems, such as muscular (primarily skeletal muscle), hematologic (anemia), cardiovascular (clinical and/or subclinical), and endocrine (decreased insulin-like growth factor-1 [IGF-1], decreased DHEA-S, and insulin resistance, etc.) systems [30, 50–54]. It is conceivable that systemic inflammation could contribute to frailty through its detrimental effects (functional impairment and/or structural damage) to these organ systems. In fact, studies have shown that circulating IL-6 levels have inverse associations with hemoglobin concentration and IGF-1 levels in frail older adults, but not in nonfrail controls; low hemoglobin and IGF-1 levels are each independently associated with frailty, as well [30, 55]. Therefore, it is proposed that low grade, chronic systemic inflammation plays a key role in the pathogenesis of frailty, directly or through other intermediate processes (Fig. 5).

5 Conclusion and Future Direction

Frailty is a common geriatric syndrome that affects millions of older adults. The clinical presentations of frailty, as currently defined, include fatigue, low muscle strength, poor motor performance, low levels of physical activity, and weight loss, emphasizing patient's physical function and performance. Its cardinal feature includes the involvement of multiple physiologic organ systems, decreased physiologic reserve, and increased vulnerability to stressors. Frail older adults have a chronic systemic inflammatory phenotype marked by increased levels of common molecular and cellular inflammatory markers, IL-6, CRP, and WBC and its subpopulations, above and beyond age-related elevation. Emerging evidence suggests that this low grade, chronic systemic inflammation is a key pathophysiological fac-

tor, contributing directly or through other intermediary processes to frailty in older adults.

Given the heterogeneity of the older adult population and complexity of the frailty syndrome, additional clinical and translational studies at the population, clinical, cellular, molecular, and genetic levels are much needed to further elucidate the role of inflammation in the pathogenesis of frailty. Such efforts have begun to emerge, such as investigations into monocytic gene expression focusing on the inflammatory pathway (Leng S, unpublished and ongoing studies). In the future, potential interventional strategies targeted to the inflammatory pathways for the prevention (or delay) and treatment of the frailty syndrome could be developed. For example, if CCR5+ T-cells prove to play a critical role in the development of frailty, anti-CCR5 based therapy, a novel treatment modality for HIV infection currently under active research [56], would be a promising candidate for therapeutic development. Similarly, anti-IL-6 or IL-6 receptor modulating strategies could also be applied. With the global aging at an unprecedented rate, it is critical to advance our knowledge in the pathogenesis of frailty and to develop interventional strategies for this syndrome. It is equally important to intervene immunosenescence and improve immune function in this most vulnerable older adult population.

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CMV Infection and Frailty: Immunologic Consequences and Disease Pathogenesis

George C. Wang and Jeremy Walston

Contents

Abbreviations

CI	Confidence interval
CMV	Cytomegalovirus
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
NF-κB	Nuclear factor-kappa B
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

Keywords: Cytomegalovirus • Frailty • Inflammation • Immunosenescence • T-cells

G. C. Wang (✉) · J. Walston
Division of Geriatric Medicine and Gerontology
Department of Medicine
Johns Hopkins University School of Medicine
5505 Hopkins Bayview Circle
John R. Burton Pavilion
Baltimore, MD 21224
Tel.: 410-550-0546; 410-550-1003
Fax: 410-550-2513
E-mail: gwang7@jhmi.edu; jwalston@jhmi.edu

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1 Frailty: Brief Overview

Frailty is a common geriatric syndrome that has been variously characterized as a wasting state of decreased physiologic reserve, loss of physiologic complexity, and accumulation of deficits [11, 58, 83], and is an independent risk factor for poor outcomes in older adults [7, 30, 113]. A physiologic phenomenon that has been consistently observed in frail older individuals is a generalized inflammatory state, beyond age-related changes [26, 56, 87, 106]. Frail older adults have higher levels of systemic inflammatory markers, including interleukin-6 and C-reactive protein, than older adults who are not frail, even when chronic diseases are excluded [106]. The chronic activation of inflammatory pathways is known to influence skeletal muscle mass decline, the anemia of chronic disease, hypothalamic-pituitary-adrenal axis (HPA axis) activity, cognition, and a number of chronic disease states, and likely plays an important role in the pathogenesis of frailty through its effects on these multiple physiologic systems [28].

2 Association between CMV Seropositivity and Frailty

Because chronic inflammation appears to be crucial in the development of frailty, and because cytomegalovirus (CMV) is known to trigger chronic inflammation, investigators recently designed a study to determine the relationship between CMV infection and frailty. Using a cross-sectional study design, these investigators demonstrated an association between CMV seropositivity and frailty in older women aged 70–79 [87]. The participants in this study were drawn from the Women's Health and Aging Studies (WHAS) I and II, which are two complementary population-based, prospective, observational studies involving community-dwelling older women in Baltimore, Maryland, who were randomly sampled from the Medicare enrollment file [27, 29].

Frailty in this study was defined according to validated criteria consisting of 5 measurable characteristics: shrinking, weakness, poor endurance and energy, slowness, and low physical activity level [30]. Older women meeting a critical mass of three or more components were defined as frail. Chronic CMV infection was defined as the presence of anti-CMV IgG antibodies in the plasma. These investigators demonstrated a cross-sectional association between CMV seropositivity and frailty in older women. After being adjusted for age, history of smoking, body mass index ≥25 kg/m^2, diabetes mellitus, and congestive heart failure, the odds ratio (OR) for frailty in persons with CMV seropositivity was 3.2. Furthermore, serum interleukin (IL)-6 level was an effect modifier, enhancing the association between CMV seropositivity and frailty; in persons with high IL-6 level (≥4.2 pg/mL) and CMV seropositivity, the adjusted OR for frailty was 20.3 (Table 1).

Table 1 The association between CMV seropositivity alone, and stratified by Interleukin-6 (IL-6) level, and prevalent frailty status in the women's health and aging studies I and II* (From ref. (9), with permission of the publisher.)

				Multivariate Models			
		Unadjusted (n=724)		Adjusted† (n=706)		Adjusted including‡ SES (n=706)	
Risk Factor	Frailty Status	Odds Ratio (95% Confidence Interval)	*P*-value	Odds Ratio (95% Confidence Interval)	*P*-value	Odds Ratio (95% Confidence Interval)	
1. CMV-positive	Not frail	1.0		1.0		1.0	
	Prefrail	1.5 (0.9–2.4)	.13	1.5 (0.8–2.5)	.17	1.2 (0.7–2.1)	
	Frail	3.2 (1.2–8.9)	.02	3.2 (1.1–9.2)	.03	1.8 (0.6–5.1)	
2. High IL-6‖	Not frail	1.0		1.0		1.0	
	Prefrail	1.7 (1.2–2.5)	.006	1.4 (0.9–2.1)	.09	1.4 (0.9–2.0)	
	Frail	2.9 (1.7–4.8)	<.001	2.1 (1.2–3.7)	.01	2.0 (1.1–3.6)	
3. CMV-positive, stratified by IL-6 level							
Low IL-6¶	Not frail	1.0		1.0		1.0	
	Prefrail	1.0 (0.5–1.8)	.93	0.9 (0.5–1.7)	.73	0.9 (0.3–2.9)	
	Frail	1.7 (0.5–5.4)	.39	1.5 (0.4–4.9)	.53	0.8 (0.4–1.5)	
High IL-6‖	Not frail	1.0		1.0		1.0	
	Prefrail	4.4 (1.7–11.3)	.002	5.5 (2.0–14.9)	.001	4.2 (1.5–11.3)	
	Frail	14.6 (1.8–116.6)	.01	20.3 (2.3–178.3)	.007	10.0 (1.1–90.8)	

*Two complementary cohorts of community-dwelling older women, aged 70 to 79.
†Adjusted for age, history of smoking, body mass index (BMI) ≥25 kg/m^2, diabetes mellitus, and congestive heart failure (CHF).
‡Adjusted for age, history of smoking, BMI ≥25 kg/m^2, diabetes mellitus, CHF, Caucasian race, and high school education; and education used as markers for socioeconomic status (SES).
‖Top tertile (≥4.2 pg/mL).
¶Bottom two tertiles (<4.2 pg/mL).

It should be noted, however, that in this study cohort of older women, race was a potential confounder in the relationship between CMV seropositivity and frailty. Of the CMV-seropositive older women in this cohort, 75.5% were Caucasian, while of the CMV-seronegative older women, 92.8% were Caucasian. Black race is known to be associated with frailty [30]. In fact, when race and high school education were both adjusted in the study's regression model, the association between CMV and frailty became non-significant (see Table 1). However, in those older women with a high IL-6 level, suggesting the presence of inflammation, the association between CMV and frailty remained significant (OR = 10, P=0.04) even when the models were race adjusted. Several reasonable interpretations of these results follow. First, the study might be underpowered to detect a significant association between CMV seropositivity and frailty in those women without a high IL-6 level. Second, the possible contribution of CMV to the development of frailty would be most relevant and probably only effective, in an inflammatory milieu, caused by CMV infection itself or other factors. Third, in those older adults in whom CMV infection either was unsuccessful in causing inflammation in the host or did not coexist with other inflammation-inducing factors, the host was protected from developing frailty despite their CMV seropositivity. Since this was a cross-sectional study, the direc-

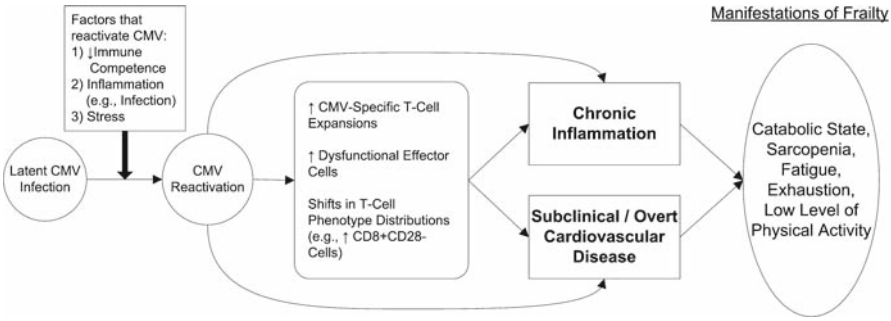

Fig. 1 Hypothesized pathogenic pathway between CMV infection and frailty

tion of causality in the relationship between CMV infection and frailty simply cannot be determined.

Other considerations regarding the role of CMV infection in the development are also in order. In this cohort, the prevalence of CMV seropositivity was 87.0%, and the prevalence of frailty was 14.3% [87]. Since there was not a one-to-one correspondence between CMV seropositivity and frailty, given the wide discrepancy between the relatively high prevalence of CMV seropositivity and the prevalence of frailty, other additional factors and unaccounted variables most likely play a role in the relationship between CMV infection and frailty [87]. Taking another, but similar, perspective on this lack of correspondence in the prevalence of CMV seropositivity and frailty, the authors of this chapter suggest that intrinsic host and extraneous factors protect certain humans latently infected with CMV from developing frailty, or, conversely, that host and extraneous factors, interacting synergistically with CMV, predispose a subset of humans with latent CMV infection to the development of frailty through detrimental immune system modulations.

In the remaining sections this chapter, attempts will be made to address the questions raised in the preceding paragraphs regarding the potential role of CMV infection in the development of frailty. Fig. 1 provides an overview of the proposed modal pathway that connects chronic CMV infection to frailty and other adverse late-life outcomes. The following sections examine the biology of CMV infection and immunologic mechanisms that lead to pathogenesis. This review will provide insights into why CMV infection causes frailty in some but not others, and will raise further questions regarding the possible role of CMV infection in the pathogenesis of frailty and attempt to answer them.

3 CMV Infection

3.1 Epidemiology

Human CMV (HCMV) is a ubiquitous virus of approximately 235 kbp linear DNA that has come to be best known by the opportunistic disease it causes in

acquired immunodeficiency syndrome (AIDS) patients and immunosuppressed transplant recipients. As with all Herpes viruses, after a generally asymptomatic primary infection by CMV, lifelong latency follows, during which the virus resides latent in myeloid progenitor cells in the immunocompetent host [80]. In epidemiologic studies, the presence of serum immunoglobulin G (IgG) to CMV constitutes evidence of prior infection by the virus and, by presumption, of latent infection.

The virus has a worldwide distribution. Prevalence varies across the world, ranging from 40% to 99% of the populations studied [15, 21, 32, 37, 42, 43, 67, 68, 84, 92, 96, 97], being lowest in Europe and the United States and highest in Africa. The prevalence is generally higher and the age of viral acquisition younger in developing countries. In studies that examined the prevalence across strata of socioeconomic status within the same geographic cohort, prevalence is consistently higher in individuals of lower socioeconomic status [13, 37, 38, 67, 96, 97]. For example, in a French cohort, seroprevalence in the low and high socioeconomic strata was 70% and 47%, respectively [37]. In 2 U.S. cohorts, seroprevalence in the low- and high-income groups was 77% and 36%, respectively, in one cohort, and 71% and 47%, respectively, in the other cohort [96, 97], although in the latter study the difference in CMV seroprevalence across income groups could be explained by other markers of socioeconomic status that were examined. This latter study is one of the largest cross-sectional seroepidemiologic studies of the prevalence of CMV infection in the United States, which uses data and samples from the National Health and Nutrition Examination Survey III, a nationally representative population-based sample [97]. The investigators reported a difference in seroprevalence across racial and ethnic groups: The age-adjusted prevalence was 51% in the non-Hispanic white, 76% in the non-Hispanic black, and 82% in the Mexican American groups. Although such a difference could possibly be attributed to socioeconomic status, this difference persisted even after data were adjusted for variables that could potentially confound the relationship between race/ethnicity and CMV seroprevalence, including age, sex, household income level, education, marital status, area of residence, census region, family size, country of birth, and type of medical insurance. It is possible that the difference in seroprevalence across racial and ethnic groups could be explained by difference in sexual behavior [97], although the study did not include data to allow such an explanation. This same study also showed that women have a higher seroprevalence (60%) than men (52%), and that those with fewer years of education, those born outside of the U.S., and those living in the South have higher CMV seroprevalence. In consistence with the previously known relationship between CMV prevalence and age, this study reported increasing CMV seroprevalence in progressively older age groups: 6–11 years, 36%; 12–19 years, 42%; 20–29 years, 49%; 30–39 years, 54%; 50–59 years, 74%; 60–69 years, 83%; 70–79 years, 89%; ≥ 80 years, 91%. Although it is possible that the age-prevalence relationship could result from the cohort effect in this cross-sectional study, the increasing prevalence with age is most plausibly explained by the cumulative exposure to the virus throughout life.

3.2 Course of Infection

3.2.1 Methods of Transmission

CMV is transmitted through contact with infectious body fluids of persons who are shedding virus (see below) but not through the airborne route. The virus can be found in milk, urine, saliva, tears, cervical secretions, semen, and blood products [4]. Accordingly, contact with young children and sexual activity are common risk factors for primary CMV infection [13, 76]. Since the virus is latent in myeloid progenitor cells and whence-derived tissue resident macrophages and dendritic cells [45, 80], transmission can also occur through transfusion of leukocyte-containing or contaminated blood products and through transplantation of solid organs or hematopoietic cells. A prior CMV infection does not prevent reinfection by a different strain of virus, as the coexistence of different strains of virus or shedding of new strains in serial examinations has been documented in both immunocompetent and immunocompromised hosts [6, 14, 16, 17, 94]. Among those without obvious immunodeficient conditions, children and persons with multiple sexual partners are more likely to have reinfection [6, 14]. It is important to note that higher levels of neutralizing antibodies appear to reduce the rate of reinfection [3]. Vertical transmission of CMV from mother to fetus is common and poses an important public health problem, especially since infected infants could shed the virus for years [64]. It can be accomplished in utero, intrapartum, and postpartum (through human milk) as a result of maternal primary or recurrent infections.

3.2.2 Infection in Immunocompetent Hosts

It is traditionally reported that CMV infection does not cause significant illness in immunocompetent hosts [18, 64]. Primary infection in otherwise healthy adults can lead to a (heterophile negative) infectious mononucleosis syndrome with fever lasting 9 to 35 days, lymphadenopathy, and relative lymphocytosis ([49], cited in [18]). Despite copious descriptions in the literature of the manifestations of CMV infection and disease in immunocompromised individuals, few reports (cited in [114]) describe symptoms in immunocompetent adults. In a recent cross-sectional study that selected immunocompetent patients based on a list of symptoms, CMV infection, defined by a CMV-specific IgM level of > 300 U/mL, in the absence of other diagnoses of viral and *T. gondii* infections, was associated with abnormal liver enzymes, malaise, sweats, fever, lymphadenopathy, and jaundice [114]. However, the prevalence of these symptoms in primary CMV infection in the immunocompetent host cannot be determined from this study, since only symptomatic patients were recruited. It remains unclear how frequent CMV primary infections remain asymptomatic and thus undetected by health care professionals. Other than reports of longitudinal outcomes in children with congenital CMV infection, few published studies have examined the long-term effects of CMV infection in immunocompetent hosts and its manifestations in older adults.

3.2.3 Latency

After primary infection, CMV, as with all Herpes viruses, remains latent in the host. Thus, the presence of serum CMV-specific IgG signifies prior primary infection and, by inference, latent CMV infection. A definitive understanding of the range of cell types that can harbor latent virus is still lacking [64]. Nevertheless, myeloid progenitor cells and whence-derived tissue resident macrophages and dendritic cells [45, 80], polymorphonuclear cells, T-lymphocytes, endothelial cells, renal epithelial cells, and salivary epithelial cells [18] may all harbor the virus. Whereas myeloid lineage progenitor cells harbor latent virus, salivary and renal epithelial cells probably harbor persistently replicating virus [64]. Although the molecular mechanisms that control latency are not well elucidated, a strong and broad T-cell response likely plays a key role in suppressing active viral replication [64, 100]. In later sections, we will address and speculate on the undesirable consequences that such a virus-directed T-cell response might have in the host. There is likely a delicate balance that needs to be maintained, in order to both suppress viral replication throughout the host's life and avoid overactivation of the immune response that might have untoward effects in the host. The disruption of such a balance might underlie the role that CMV infection plays in the pathogenesis of frailty and inflammation in older adults.

One mechanism by which CMV could remain in host cells and avoid immune detection is the downregulation of the expression of HLA Class I molecules on the surface of infected cells [8], thus crippling the ability of cytotoxic CD8+ T-cells to recognize and destroyed infected cells. However, since HLA Class I downregulation leads to increased susceptibility to lysis by natural killer (NK) cells [24], in order for CMV to successfully reside in host cells, mechanisms must and do exist by which CMV downmodulates NK-cell activity (reviewed in [63]). Whether CMV remains latent in a nonreplicating form or replicates at low levels in host cells still remains a topic of debate [64]. Of interest, CMV DNA has been detected even in the PBMC of seronegative individuals [55].

3.2.4 Reactivation

Reactivation of CMV from the latent state is well known to occur after immunosuppression or immunodeficiency, such as that in transplant recipients taking immunosuppressive regimens or patients with AIDS. On the other hand, little is known about the natural course of CMV infection in immunocompetent individuals, particularly with regard to the presence and frequency of viral reactivation from latency. Nonetheless, the molecular triggers that reactivate lytic replication are still being worked out, both in immunosuppressed and immunocompetent hosts. We will focus our discussion on mechanisms of reactivation and course of infection in immunocompetent adults, by drawing on current understanding of viral reactivation in immunocompromised individuals, and venture to speculate on the role of CMV infection in the pathogenesis of frailty.

The nature of CMV latency itself is not completely elucidated [90]. In one scenario, CMV could be truly latent in host cells. Conceivably, reactivation would then occur via an unknown stimulus, or a combination of stimuli, that serve to induce viral gene expression, leading to viral replication. Such a stimulus or stimuli could occur in healthy individuals and cause occasional (presumably asymptomatic or mildly symptom-inducing) viral reactivation. In another scenario, CMV could continuously replicate in infected cells, which are removed by immune surveillance in healthy individuals. In this case, reactivation would then result from failure of the immune system to remove these infected cells. Current evidence points to the first scenario as the likely mechanism of CMV reactivation (reviewed in [44]).

Cellular differentiation, such as that of monocytes to macrophages in vitro, has been reported to induce immediate-early (IE) gene expression, an important first step in viral replication and, thus, reactivation [91, 102]. Ex vivo differentiation of myeloid dendritic cell progenitors to mature dendritic cells is associated with IE gene expression, increased copy number of viral genome, and release of infectious virus [80]. Latency of CMV in myeloid lineage progenitor cells enables the virus to periodically reactivate and release progeny viruses as these host cells undergo their normal differentiation program, including that occurring during natural infections (see below) [90].

From a different perspective, further speculation on the likely nature of the stimulus or stimuli that lead to viral reactivation can be informed by examining the allogeneic response. In allogeneic transplant recipients, cytokine expression resulting from the allogeneic response leads to activation of NF-κB and AP-1, which have been shown to be important transcription factors that induce IE gene expression, a process that is likely one of the first steps in CMV reactivation (reviewed in [44]). Specifically, TNF-α has been well demonstrated to play an important role in CMV reactivation, via the induction of CMV IE gene expression [79]. An allogeneic response to a transplanted organ that leads to CMV reactivation closely resembles a natural inflammatory immune response to infection [44]. Thus, in an immunocompetent individual, it is conceivable that infections, through eliciting an immune response that includes TNF-α and IFN-γ expression, can lead to reactivation of CMV [44]. In addition, the differentiation of myeloid lineage cells in response to natural infections allows CMV to be reactivated in these cells. From an evolutionary standpoint, it is advantageous for CMV to reactivate from a latent state when the host is infected, so that CMV can escape from a host who might die from the new infection [44].

Little is known about the temporal frequency of CMV reactivation in healthy, immunocompetent carriers. Asymptomatic shedding into urine and saliva is said to occur periodically in healthy carriers [64, 90], but the authors of this chapter are not certain whether CMV shedding into the urine or saliva, as a result of persistent CMV replication in epithelial cells [64], has the same immunologic consequences as true reactivation from a latent state, such as that in myeloid lineage cells. Few reports in the literature address CMV reactivation in immunocompetent adults. Most of these reports examined the phenomenon of CMV reactivation at one time point or within a very short period of time, not long enough to be considered a longitudinal time frame [61, 62, 66, 98]. Although these studies reported detection of “CMV reactivation” in healthy individuals, the definitions of CMV reactivation varied, some using serum

IgM titers and/or IgG titers (definitions which might not reflect true reactivation) [61, 66] and some using CMV DNA detection in urine [57, 62, 98]. In one study, CMV DNA was detected in the urine of 10 of 11 healthy elderly individuals and none of 31 young controls over a 6-month period [98]. Another study examined CMV DNA in 4 urine collections over a 14-month period and, in contrast to the results of the aforementioned study, detected CMV DNA in 4 out of 13 healthy individuals younger than 40 and none of 17 individuals older than 40 [57]. In both of these studies, it is unclear how frequent CMV DNA was detected over the study period mentioned above. In another cross-sectional study, urine CMV DNA was detected in a higher percentage of healthy astronauts (15 out of 71) than age-matched controls (1 out of 61). Although this result could suggest an association between stress, such as that from spaceflight, and CMV reactivation, stress hormone levels were similar in those who shed CMV in the urine and those who did not [62].

To date, there is no long-term study that examines the temporal frequency of CMV reactivation in immunocompetent hosts. Given the current understanding of CMV latency and reactivation, the detection of CMV DNA in blood samples devoid of cellular components, such as plasma or serum, would serve as strong evidence of true CMV reactivation from a latent state, capable of eliciting an immune response from the host. It is important to understand how frequently true CMV reactivation occurs in immunocompetent adults, since CMV reactivation could repetitively elicit immune responses that, over time, result in deleterious effects on the host. We will address this consideration again in later sections when we discuss the effects of CMV infection on the immune system and speculate the mechanism by which CMV could lead to frailty.

Weakened cellular immunity in immunocompromised hosts is associated with frequent CMV reactivation. Aging, by producing a diminished cell-mediated immunity, could lead to an increased frequency of CMV reactivation. The parallel increased rates of reactivation of varicella-zoster virus in both immunocompromised persons and apparently immunocompetent older adults support this hypothesis [35]. In apparently immunocompetent older adults, there is evidence of decreased cell-mediated immunity to varicella-zoster with age demonstrated by delayed hypersensitivity skin test [12]. More specifically, therefore, just as latent varicella-zoster virus is not reactivated in all older adults [20], frail older adults could represent a subset of aged humans who, as a result of intrinsic or extraneous factors, have a more profound diminishment in cell-mediated immunity and, thus, are more likely than non-frail older adults to experience more frequent CMV reactivation and its corresponding consequences.

3.3 Immune Responses and Inflammation in CMV Infection

CMV has evolved to adapt well to the host immune response in 2 significant ways. First, it has developed many strategies to evade the host immune response. Second, it turns around and exploits the host immune response to its benefit, facilitating its

replication and dissemination [63]. We have already discussed previously that the transcription factors, NF-κB and AP-1, activated in the course of an inflammatory response in fact induce IE gene expression and trigger CMV replication. In addition, chemokines encoded by CMV recruit monocytes to the site of CMV infection, which then serve as host cells that enable dissemination of the virus [86].

The immune response against CMV plays a very important role in limiting reactivation at local sites where it occurs, thus preventing the dissemination of CMV and widespread infection [90]. In this section, we discuss immune responses observed in HCMV infection and consider the potential pathogenic effects that such responses could have in the long term, especially speculating on the case of frail older adult.

In primary infection, anti-CMV IgG appears 2 to 3 weeks after the onset of symptoms, and CMV IgM remains detectable for four to six months [114]. Of note, antibody titers increased over time in CMV shedders [62], suggesting that there is a persistence of immunogenic stimulus from the CMV possibly from episodic reactivation. The observation that CMV antibody titers are higher in individuals of older age [61, 98] also suggests that long-term latent infection, through episodic reactivation, leads to higher CMV antibody titers.

A strong T-cell response plays a crucial role in keeping CMV reactivation in check throughout the life of the host [64]. It has been observed that, in CMV infection, the frequency of CMV-specific T-cells, often existing as oligoloncal expansions, can reach 25% or more of the CD8+ pool [34, 47, 54]. CMV-specific CD45RA+CD27-CCR7- effector T-cells expand when stimulated by their cognate peptide in the presence of helper T-cell-derived cytokines [103]. In addition, these reactivated effector T-cells change their surface phenotype from CD45RA to CD45RO and regain CCR7, but at the same time still maintain their effector function. Taken together, these data can support a cogent model in which CMV-specific effector T-cells can maintain latency by killing rare virus-expressing cells. When CMV reactivates, these CMV-specific T-cells can carry out cytotoxic function directly, rapidly change into effector memory cells, and expand to produce an abundant number of progeny to meet the challenge of a higher viral load. In fact, it has been shown longitudinally in immunosuppressed individuals that CMV reactivation leads to an increase in the percentage of CMV-specific effector CD8+ T-cells (or both an increase in percentage and a shift in phenotype dominance to a CD27- effector phenotype in individuals who start off with a CMV-specific CD27+ memory phenotype) that persists more than six months after viral reactivation [31]. There is also a strong correlation between the percentage of CMV-specific CD8+ T-cells and the percentage of CD27- cells within these cells [31]. This latter observation suggests that in individuals in whom these is a predominance of CMV-specific CD27- effector T-cells, CMV reactivation was probably a recent event, or, more generally, occurs more frequently.

This model of understanding of CMV reactivation suggests that, if CMV infection plays a role in the pathogenesis of frailty, frequent reactivation of CMV could lead to an accumulation of expanded CMV-specific effector T-cells and their persistence, which have been associated with poor outcomes in older individuals [39].

3.4 CMV Infection and CMV-Induced Immune Responses Associated with Aging

Whereas varicella zoster virus reactivation leads to higher frequencies of a clinically apparent disease, shingles, in older age [20], CMV reactivation, if it does occur more frequently in older age, does not lead to any currently identifiable disease or syndrome in immunocompetent, healthy individuals.

3.4.1 Alterations in T-cell Subset Distribution Associated with CMV Infection

Other chapters of this book detail alterations in the phenotypic distribution of T-cell subsets that are observed in older age. In this section, we will discuss the observations regarding T-cell activation and replicative senescence that have been specifically associated with CMV infection.

Investigators have repeatedly observed that increased numbers of CD28- T-cells are found in persons of older age [23] and that clonal expansions of CD8+CD28- T-cells accumulate in older adults [78]. For further discussions on CD28- T cells, see chapter 17. The fact that these increased numbers of CD8+CD28- T-cells possessed high anti-CD3 redirected cytotoxic activity suggested that in older humans these cells represent armed effector cells targeting self cells harboring intracellular pathogens [23]. Many studies have repeatedly demonstrated associations between CMV infection and such presence of increased numbers of CD8+CD28- T-cells in older individuals [59, 71]. In fact, the CMV-associated alterations in the phenotypic distribution of CD8+ T-cell subsets appear to be similar in the very young and the very old [51, 71].

Age-related changes in the immune system have been examined in the context of CMV seropositivity [59]. Using regression analysis, investigators in the cited study were able to show that when the concentration of CD4+CD28- or CD8+CD28- T-cells was adjusted for CMV seropositivity, the number of CD28- T-cells was no longer statistically associated with age. That is, when CMV seropositivity was taken into account, older age was no longer associated with increased numbers of CD4+CD28- or CD8+CD28- T-cells. At the same time, within both the elderly and young cohorts, CMV seropositivity was associated with marked increases in the concentrations of CD4+CD28- and CD8+CD28- T-cells.

In a large cohort of Austrian older adults, of whom 35% were CMV-seronegative, latent CMV infection was associated with a significant increase in the percentage of CD28- T-cells (defined as effector cells by the authors) within the CD8+ T-cell pool (48.4 ± 1.4 in CMV-seropositives vs. 25.2 ± 1.6 in CMV-seronegatives) [108]. This increase appeared to derive directly from parallel decreases in the CD28+CD45RA+ and CD28+CD45RA- subsets (defined as naïve and memory cells, respectively, by the authors) within the CD8+ T-cell pool. Similar changes in the same directions were observed in CD4+ T-cell subsets as well. Unfortunately, these observations

were not adjusted for age. Therefore, it cannot be determined whether immunologic changes driven by or induced during the aging process confound the CMV-associated changes that were reported in this cohort.

3.4.2 Phenotype and Function of CMV-specific T-cells in Older Persons

In older individuals, CMV-specific CD8+ T-cells have a highly polarized surface phenotype characteristic of effector memory cells (CD28-, CD57+, CCR7-) [48]. In young individuals, a significant portion of CD8+ T-cells specific for HLA-A2 restricted CMV pp65 peptide has a phenotype characteristic of naïve T-cells (CCR7+CD45RA+). In contrast, in old individuals, most of the CMV-specific CD8+ T-cells have a phenotype characteristic of effector-memory T-cells ($CCR7^{null}$ $CD45RA^{null}$ or $CCR7^{null}$ CD45RA+) (reviewed in [50]). Thus, the distribution of the phenotypes of the cells within the CMV-specific CD8+ T-cell population is significantly different in young and old individuals.

The number of functional CMV-specific CD8+ T-cells (namely, cells that produce IFN-γ when challenged with HLA-restricted CMV peptide) is comparable in young and old individuals [72]. However, older individuals have a markedly higher number of dysfunctional CMV-specific CD8+ T-cells. These dysfunctional cells are anergic and do not respond to stimulation with specific antigen *ex vivo* [73,74]. Thus, the overall increase in the number of CMV-specific CD8+ T-cells in older individuals results from an accumulation of such dysfunctional CMV-specific CD8+ T-cells.

It has been suggested that increased CMV viral load in older age could potentially be the underlying factor that drives an increased number of CMV-specific CD8+ T-cells in older persons [50]. However, no direct evidence is currently available to support this reasonable speculation.

Much less is currently known about the number, phenotype, and function of CMV-specific CD4+ T-cells in older individuals and the relationship of these cells with the outcome and survival of older persons [50].

3.4.3 Accumulation of CMV-specific T-cells in Older Adults

CMV reactivation may drive accumulation of CMV-specific T-cells. Since CD8+CD28- T-cells, driven to differentiation from CD8+CD28+ T-cells by antigen stimulation, are resistant to apoptosis [77], it is understandable that the resultant CD8+CD28- CMV-specific effector T-cells will tend to accumulate with time.

In immunodeficient persons infected with the HIV, the restoration of immune protection against CMV after recovery from CMV disease was characterized by a broad and diverse antigenic repertoire and CMV-specific T-cells displaying an "early" (CD8+CD27+CD28+) and "intermediate" (CD8+CD27-CD28+) differentiation phenotype [85]. By extension of this observation, the accumulation of CMV-specific T-cells with a late differentiation phenotype (CD8+CD28-) in older

adults could render them less capable of controlling their CMV infection and, consequently, the pathologic manifestations of the infection.

Frail older individuals have an increased number of CD8+CD28- T-cells compared with older individuals who are not frail [88]. It is not known whether the increase in the number of CD8+CD28- T-cells in frail older individuals is CMV-driven. Since not all CMV-seropositive individuals are frail, we can only speculate that in those CMV-seropositive individuals who are or become frail, an increased number of CMV-specific CD8+CD28- T-cells, which are most likely replicatively senescent and apoptosis resistant [77], could potentially underlie or contribute to the pathogenesis of frailty.

In a study that examined T-cell clone numbers in the context of latent CMV infection, CMV-seropositive nonagenarians have a higher number of T-cell clones than CMV-seronegative nonagenarians (mean clone number 22.6 vs. 7.4) [39]. This association was not found in middle-aged individuals. The number of clones in the middle-aged individuals was similar to the number in CMV-negative nonagenarians. Thus, these results imply that long-term latent CMV infection seems to have contributed to the higher number of T-cell clones in older adults in this study. A definitively conclusion, of course, can only be obtained from a true longitudinal study.

Indeed, there is evidence that CMV reactivation results in higher numbers of CMV-specific T-cells. Following allogeneic stem cell transplantation, higher frequencies of CMV-specific CD8+ and CD4+ T-cells were observed in persons with CMV reactivation, measured by CMV antigenemia [75]. Thus, the higher frequency of CMV-specific CD8+ T-cells observed in nonagenarians compared with middle-aged adults [39] is likely the result of the cumulative effects of episodic reactivation in lifelong latent CMV infection.

3.5 CMV, Cardiovascular Disease, and Frailty

3.5.1 CMV Infection and Cardiovascular Disease

Multiples lines of evidence have implicated a role for CMV infection in the development of cardiovascular diseases (CVD). These include results from epidemiologic studies, pathological examinations of atheromatous lesions, *in vitro* mechanistic studies utilizing cell systems, and experimental animal models. Because frailty has been demonstrated to be associated with subclinical cardiovascular disease [69], it is reasonable to speculate that CMV infection could contribute to the development of frailty through the intermediary pathway of subclinical cardiovascular disease, a condition characterized by abnormalities on noninvasive testing, such as carotid ultrasound, ankle-brachial index, electrocardiogram, and echocardiogram, and lack of a diagnosis of clinically manifest CVD [53]. Examples of clinically manifest CVD include coronary heart disease (CHD), acute coronary syndrome (ACS), myocardial infarction (MI), congestive heart failure (CHF), transient ischemic attack (TIA), stroke, and intermittent claudication.

Epidemiologic studies investigating the role of CMV in CVD utilize different clinical outcomes along the spectrum of disease progression, severity, organ distribution, and disease context (e.g., native atherosclerosis vs. post-procedural restenosis). Thus, caution should be heeded in interpreting the results and comparing different studies. Seroepidemiologic studies have demonstrated associations between CMV seropositivity and atherosclerosis [1, 9, 70, 93], while other have not [2, 60]. Reasons for such discrepancies include inadequate statistical power and heterogeneous definitions of clinical outcomes. However, the incremental increase in odds of disease with incremental increase in CMV antibody titers reported in some studies strengthens the plausibility of the associations. Many of these epidemiologic studies examine restenosis after coronary angioplasty or vascular procedures, scenarios in which disease pathogenesis might differ from that of native coronary atherosclerosis [19]. A strong evidence for a causative role of CMV in native coronary atherosclerosis comes from a prospective study in which the highest titers of CMV antibody were associated with the development of incident CHD in those previously free of the diagnosis [93]. Importantly, in this study lower CMV antibody titers were not associated with CHD. This fact is consistent with the conceptual framework we are developing in this chapter that more frequent CMV reactivation, leading to higher antibody titers, contribute to clinically manifest outcomes, including frailty and cardiovascular disease, through similar, overlapping, or distinct pathogenic mechanisms. We have not cited many other studies in which CMV infection in atherosclerosis was studied in the context of cardiac transplants, since confounding factors, such allogeneic responses, could complicate interpretation of the results for the sake of our discussion.

Prospective studies on the association between CMV seropositivity and MI and CHD death, later stages in the progression of atherosclerotic disease severity, showed no increased risk for these events in CMV-seropositive middle-aged individuals [81, 82] and older adults [89]. It should be noted, however, that these studies examined CMV seropositivity solely as a dichotomous variable and did not measure CMV antibody titers quantitatively. In fact, in a cross-sectional study examining CMV seropositivity and the risk of MI in young persons, CMV seropositivity itself was not associated with premature MI, but when CMV antibody titers were measured quantitatively in the same population, an anti-CMV IgG titer ≥ 100 EU/mL was associated with an increased risk of premature MI independently of age, sex, smoking status, history of hyperlipidemia or hypertension, educational level, and occupation [33].

Other types of studies have shed light on the possible pathogenic mechanisms of CMV infection in CVD. Pathological examinations of atheromatous lesions show a higher likelihood of detecting CMV in atheromatous than normal vessles (reviewed in [19]). This finding is more pronounced when a sensitive method, such as detection of viral genome by PCR, is employed to detect latent CMV in arterial specimens [41]. In murine models, MCMV infection could lead to atherogenesis through 2 mechanisms, accumulation of inflammatory cells and increased serum low-density lipoprotein cholesterol (reviewed in [19]). HCMV can infect arterial smooth muscle cells and induce the migration of these cells, promoted by expression of the viral

chemokine receptor, US28 [99]. The IE gene products of HCMV could increase vascular smooth muscle cell proliferation [101]. In a functional study, CMV-seropositive individuals have impaired vascular response to vasodilators consistent with endothelial dysfunction [36]. Finally, CMV-seropositive patients with CHD have increased numbers of CD8+CD28- T-cells compared with CMV-seropositive healthy controls. Interestingly, this finding parallels the increase in the number of CD8+CD28- T-cells in frail compared with nonfrail older adults [88] and invites the speculation that similar or analogous immunologic mechanisms could underlie the pathogenesis of CVD and frailty, at least in a subset of individuals.

3.5.2 Subclinical Cardiovascular Disease and Frailty

Frailty has been associated with clinically manifest CVD and, perhaps more importantly, subclinical CVD. It should be noted that many older adults with clinically manifest CVD are not frail, and older adults with subclinical CVD but no clinically manifest CVD can be frail [69]. The association of frailty with subclinical cardiovascular disease is important because it contributes to the current conceptualization of frailty as a clinical syndrome in which physiologic declines and subclinical processes culminate in a critical mass of physiologic disturbances and loss of physiologic complexity that leads both to the physical manifestations of frailty and to the poor outcomes predicted by the diagnosis [58, 83, 105]. In addition to its suggested role in the development of manifest cardiovascular disease, CMV infection could contribute to the development of subclinical cardiovascular disease; increasing levels of CMV antibody titers have been associated in a graded relation with carotid intimal-medial thickening, a measure of subclinical atherosclerosis [70]. Thus, it is conceivable that, through periodic reactivation of latent infection, leading to higher levels of antibody titers, long-term CMV infection could result in subclinical atherosclerosis, which, in turn, could contribute to the development of frailty in the older adult.

4 Summary: Future Directions in CMV and Frailty Research

There is considerable evidence that chronic CMV infection as described above plays an important role in immune system modulation later in life. This evidence provides important rationale for developing further studies that might help to establish causality between CMV and late life adverse health outcomes. However, the current evidence showing an association between CMV infection and frailty presented above is cross-sectional in nature and can not prove causality. In order to determine if CMV plays a role in the pathogenesis of frailty, further studies will be necessary to determine whether CMV causes the inflammation in frailty or whether the immunologic alterations in frailty allow CMV to reactivate and thrive and cause low level disease in older adults. In addition, further research regarding the various possible points

along the possible pathogenic pathway that connects CMV with frailty are needed, as well as population studies with longitudinal measurements establishing temporal relationships between CMV infection and frailty as represented in Fig. 1.

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Osteoporosis

Osteoporosis, Inflammation and Ageing

Lia Ginaldi, Lucia P. Mengoli and Massimo De Martinis

Contents

Abstract: Osteoporosis is substantially an age-related condition characterized by low bone mass and increased bone fragility, putting the patients at risk of fractures, which are major causes of morbidity and mortality in older people. Although ageing and estrogen deficiency are probably the 2 most important risk factors, osteoporosis can occur in any age of life. There are a large number of risk factors for the development of senile osteoporosis. Osteoporosis is currently attributed to various endocrine, metabolic and mechanical factors. However, recent discoveries suggest that these risk factors could exert their effects through immunologically mediated modulation of bone remodelling. Emerging clinical and molecular evidences suggest that inflammation exerts significant influence on bone turnover, inducing osteoporosis. Currently, growing understanding of bone physiology suggests that factors involved in inflammation are linked with those critical for bone remodelling process. Numerous proinflammatory cytokines have been implicated in the regulation of osteoblasts and osteoclasts, and a shift towards an activated immune profile has been hypothesized as important risk factor. Chronic inflammation and the immune system remodelling characteristic of ageing may be determinant pathogenetic factors. Inflamm-ageing itself plays a role in bone remodelling through proinflammatory cytokines, together with other more recently discovered immunological mediators and transcription factors. Senile osteoporosis is an example of the central role of immune-mediated inflammation in determining bone resorption.

L. Ginaldi (✉) · L. P. Mengoli · M. De Martinis
Department of Internal Medicine and Public Health
University of L'Aquila
Coppito – 67100 L'Aquila – Italy
Tel. and Fax number: +39 0861 211395
E-mail: liaginaldi@alice.it

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1 Introduction

Osteoporosis is a systemic pathology of the skeleton characterized by loss of bone mass, decreased bone mineral density and loss of microarchitectural integrity, leading to increased fragility and consequent risk of fractures. It is commonly considered an age-related disorder, representing a major cause of morbidity and mortality in older people, together with other age-related diseases, such as atherosclerosis and neurodegenerative disorders. Actually, in most developed countries, the human lifespan is greatly increased and osteoporosis is therefore becoming an emerging public health problem. Osteoporosis is fundamentally an asymptomatic condition until the appearance of a bone fracture presenting itself as a complication with clinical visibility and often lifethreatening, similar to the tip of an iceberg whose economic costs regarding public health care and rehabilitation are often incisive. Everything before the fracture has remained long unknown and it is only recently that the better understanding of bone physiology is clarifying its pathogenesis.

Osteoporosis is viewed as a heterogeneous condition which can occur in any age of life and its aetiology is attributed to various endocrine, metabolic and mechanical factors (abnormalities of parathyroid hormone and calcitonin secretion, insufficient vitamin D and calcium intake, postmenopausal hormonal condition, pregnancy, nutritional disorders, immobility and consumption of drugs such as cortisone, among others) [57]. Ageing and estrogen deficiency are probably the two most important risk factors in developing senile osteoporosis. Currently, the emerging discipline of osteoimmunology is providing a new reading register of senile osteoporosis in the light of immunosenescence and inflamm-ageing. In this chapter we will focus on the interaction between bone and immune system, considering osteoporosis as an immune mediated disease with a chronic inflammatory background.

2 Inflammation and Osteoporosis: Clinical Links

Recently, growing understanding of bone physiology suggests that factors involved in inflammation are closely linked with those critical for bone remodelling process; supporting the theory that immunosenescence significantly contributes to the aetiopathogenesis of osteoporosis. But can we really consider senile osteoporosis as an immune mediated disease or at least the result of an inflammatory process? Daily clinical practice provides the first answers and the new concept of an immune mediated mechanism at the basis of osteoporosis is clearly emerging. In particular, chronic inflammation and the immune system remodelling characteristic of various immunological diseases commonly associated with osteoporosis, may be determinant pathogenetic factors.

For example, we can often verify coincidence of systemic osteoporosis with periods of systemic inflammation as well as colocalization of regional osteoporosis with areas of regional inflammation [41, 135, 40, 30]. In the postmenopausal

period there is coincidence of inflammation with osteoporosis [82]. There is an increase in the risk of developing osteoporosis in various inflammatory conditions [50, 20, 76]. Immunological dysfunctions, autoimmune and chronic inflammatory diseases [120, 74], HIV infection [6, 77], hyper-IgE syndrome [72], inflammatory bowel diseases [80], rheumatic disorders, such as rheumatoid arthritis [58], and lymphoid neoplastic diseases [1], in particular myeloma for the B lineage and adult T-cell leukaemia lymphoma for the T lineage, are associated with osteoporosis.

Erosions seen in conditions such as rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, are typically associated with inflammation in the joints. Proosteoclastic cytokines, such as tumour necrosis factor (TNF)-α, interleukin-1 (IL-1) and IL-6, are elevated in these conditions and local cytokine profile is consistent with the cytokines that modulate bone resorption [80, 15]. An association between circulating high sensitive C reactive protein (hsCRP) level and bone mineral density (BMD) has been observed in several immune and inflammatory diseases, as well as in healthy individuals, suggesting a relationship between subclinical systemic inflammation and osteoporosis [38, 67]. The mechanisms linking hsCRP and bone metabolism are not clear, but activated inflammatory cytokines are likely involved. Inflammatory processes can up-regulate many cytokines, such as IL-1, IL-6 and TNF-α, which strongly stimulate CRP production from the liver [124, 133] as well as induce bone resorption and decrease BMD, measured at femoral neck and lumbar spine using dual energy X-ray absorptiometry [78] or ultrasonographic densitometry at calcaneous or wrist [2]. In support of this hypothesis, the production of IL-1, IL-6 and/or TNF-α by peripheral blood monocytes is positively correlated with bone resorption or spinal bone loss in healthy pre and postmenopausal [26, 97] women and serum IL-6 concentration predicts femoral bone loss in healthy postmenopausal women [102]. In addition, serum concentrations of IL-6 and TNF-α are positively correlated with serum hsCRP levels in healthy subjects [134] and those with myocardial infarction [87]. Similarly, there is a significant inverse correlation between erythrocyte sedimentation rate values and T-score, even in the absence of overt diseases. The T-score is an evaluation index of bone mineral density representing the difference in standard deviations from the mean value for normal young adults [78].

Rheumatoid arthritis (RA) is a typical example of the link between inflammation and osteoporosis. Bone loss in RA occurs both in the joints and throughout the skeleton as a result of the release of proteinases (metalloproteinases) and proinflammatory cytokines (IL-1, TNF-α), which are responsible for cartilage and bone destruction. As a result, disease activity is an independent risk factor for osteoporosis in RA [58, 95].

Particularly interesting, although less immediately evident, is the link between immunity and osteoporosis in advanced age, in which other well-known causes of bone resorption are also present, for example dysmetabolisms, decreased level of sexual hormones, nutritional deficits, decreased physical activity, age-related diseases, hyperparathyroidism, consumption of bone resorbing drugs, etc. [29, 113]. However, a more careful reading of osteoporosis reveals how the peculiar age-related immune system remodelling itself represents the most important pathogenetic factor for senile osteoporosis too.

Inflamm-ageing, i.e., the chronic inflammatory status which characterizes ageing [35, 39], represents the background underlying a wide range of age-related diseases which share an inflammatory pathogenesis. Numerous studies have shown that many cytokines, including IL-6, TNF-α and IL-1, are elevated during senescence, and play direct roles in the pathogenesis of osteoporosis too [18, 81].

3 Immune Regulation of Bone Turnover

Osteoblasts (OBs), specialized in new bone formation, are the precursors of the structural cells of the bone, that is the osteocytes. Osteoblasts in turn derive from a mesenchimal stem cell that can also differentiate into bone-marrow stromal cells and adipocytes. Osteoclasts (OCs) are on the contrary multinuclear giant cells specialized in bone resorption by the production of lysosomal enzymes. They stem from a myeloid precursor which also gives rise to macrophages and dendritic cells, which are antigen presenting cells [105, 113].

In the complex scenario of osteoimmunology, that is the immune regulation of bone turnover, the T-lymphocyte has the main role [111, 131]. The skeleton is physiologically in a state of dynamic equilibrium between new bone formation mediated by osteoblasts and resorption mediated by osteoclasts. Both these processes are finely tuned by cytokines and growth factors. Dendritic cells, specialized to present antigens, and osteoclasts, specialized to resorb bone, share the same bone-marrow precursors of the monocyte lineage and exhibit parallel lifecycles, regulated by a variety of cytokines. Release of cells into the circulation from the bone-marrow and homing from the blood stream to peripheral tissues where the immature osteoclast precursors (OCPs) differentiate into mature osteoclasts are complicated processes involving adhesion molecules, cytokines and chemokines. OCPs migrate along chemokine gradients. Stromal cell-derived factor-1 (SDF-1) produced by bone-marrow stromal cells and endothelium, has chemotactic effects on OCPs. Transforming growth factor-β (TGF-β) down-regulates the expression of SDF-1. In chronic inflammatory conditions increased cytokine levels in blood may feedback to bone-marrow to stimulate the egress of myeloid/OCPs. A major function of OCPs is to serve as a pool of progenitors for downstream effector cells, depending upon the cytokines and growth factors implicated. They differentiate into CD11c+ dendritic cells in the presence of granulocyte/monocyte-colony stimulating factor (GM-CSF) plus IL-4 but form tartrate resistant acid phosphatase (TRAP)+ osteoclasts if exposed to RANKL (receptor activator of nuclear factor κB ligand) and macrophage-colony stimulating factor (M-CSF) [64, 92]. The dendritic cells produce cytokines and chemokines directly or activate T-lymphocytes to indirectly promote osteoclasts and inflammation.

The main signalling pathway in bone resorption is mediated by the stimulation of RANK receptor on osteoclasts and their precursors by its specific ligand RANKL, predominantly expressed on osteoblasts and stromal cells. This receptor system pertains to TNF-family molecules and is essential for the development and

activation of osteoclasts. A central role in this system is also played by the ligand osteoprotegerin (OPG), competitive inhibitor of RANKL, also known as osteoclastogenesis inhibitory factor, which functions as a soluble decoy receptor to RANKL [95, 96]. Inhibition of RANKL function via OPG prevents bone loss. Other costimulatory immune receptors also exist, for example osteoclast-associated receptor (OSCAR), triggering receptor expressed in myeloid cells (TREM-2), and others [11, 33, 46]. These factors act cooperatively with RANKL in enhancing osteoclastogenesis. Intriguingly, immune cells also express RANKL. In the immune system, RANKL is expressed by activated T-cells, B-cells and dendritic cells. Therefore activated T-lymphocytes could directly induce osteoclastogenesis through RANKL [64, 92, 107].

Following antigen recognition, T-cells become activated and produce RANKL that induces osteoclast differentiation and activation. Both these processes could be downregulated by the decoy receptor OPG. In addition, they produce inflammatory cytokines, such as TNF, IL-1, IL-6, which induce osteoblasts to further express RANKL. All of these lead to an imbalance between bone formation and resorption, with consequent osteoporosis.

Osteoblasts not only play a central role in bone formation by synthesizing multiple bone matrix proteins, but regulate osteoclast maturation by soluble factors and cognate interaction, resulting in bone resorption. Osteoclast maturation requires stimulation by RANKL expressed on osteoblasts, and cognate interaction mediated by firm adhesion via inter-cellular adhesion molecule (ICAM)-1. Proinflammatory cytokines such as IL-1 and TNF-α favour bone resorption via the induction of RANKL and ICAM-1 on osteoblasts. These inflammatory signals originate from the immune system, and such immunological signals to the bone are transmitted primarily via osteoblasts to induce osteoclast maturation, resulting in secondary osteoporosis [108]. As a consequence, there is an increased stromal/osteoblastic cell-induced osteoclastogenesis during aging. Also stromal/osteoblastic cell expression of M-CSF, in association with RANKL, regulates osteoclastogenesis. Ageing is accompanied by decreased OPG and increased TNF-α, IL-1, RANKL and M-CSF expression, increased stromal/osteoblastic cell-induced osteoclastogenesis, and expansion of the osteoclast precursors pool. These changes correlate with age-related alterations in the relationship between osteoblasts and osteoclasts in bone [22]. Recently, it has become evident that the activity of immune cells affects the balance of bone mineralization and resorption carried out by the opposing actions of osteoblasts and osteoclasts. For example, increased bone resorption resulting in lytic bone lesions and osteoporosis is observed in many inflammatory and autoimmune diseases. Bone destruction is also common in many cancers, both those that reside in the bone like leukaemias and multiple myeloma, and those that metastasize to the bone such as breast and prostate cancers [64, 89, 110]. Dendritic cells, specialized to present antigens, and osteoclasts, specialized to resorb bone, exhibit parallel lifecycles. Dendritic cells arise from multipotent precursors of the monocyte lineage and are essential organizers of immune responses. They are highly specialized cells that capture antigens in peripheral tissues, migrate to lymphoid organs, and organize T-cell responses [13]. Osteoclasts are derived from the same

precursors in response to interactions with osteoblasts and other bone stromal cells. Upon differentiation into mononuclear osteoclasts and subsequent maturation and fusion into multinucleated cells, osteoclasts actively resorb bone [118]. These processes are dependent on a variety of cytokines, transcription factors and inflammatory mediators. The parallel lifecycles of these myeloid-derived cells has led to the observation of many molecular and cellular interactions between the bone and the immune system, which has been termed osteoimmunology [10].

4 Immunosenescence and Osteoporosis Share Similar Immune Profile

It is the activated immune profile which, through inflammation and inflammatory cytokine production, modulates osteoblast and osteoclast activity leading to osteoporosis. In many pathological and paraphysiological conditions, maintenance and amplification of inflammatory reactions lead to osteoclastogenesis and increased risk of fractures. The inducer cells in this process are immune cells, such as activated macrophages and lymphocytes, which produce cytokines and soluble mediators able to stimulate osteoclast differentiation and activation. Molecules that regulate osteoclastogenesis are in fact key factors in many immunological functions.

Immunosenescence is the consequence of the continuous attrition caused by lifelong antigenic load which is responsible for the chronic immune system activation and hyperproduction of proinflammatory cytokines. Therefore osteoporosis and immunosenescence share the same immunological cell and cytokine mediators.

Thymic T-cell production declines rapidly with advancing age, conditioning the peripheral immune phenotype of elderly people and subjects with senile osteoporosis. Moreover, multiple mechanisms, including antigen-driven clonal expansion and homeostasis-driven autoproliferation of postthymic T-cells, impose replicative stress on T-cells and induce the biological program of cellular senescence with characteristic phenotypic changes. T-cell immunosenescence is associated with profound changes in T-cell functional profile and leads to accumulation of CD4+T-cells which have lost CD28 but have gained killer immunoglobulin-like receptors (KIRs), markers of natural killer cells. They also exhibit cytolytic capability and produce large amounts of proinflammatory cytokines [128].

The increased production of proinflammatory cytokines with ageing derives from a chronic hyperactivation of macrophages and dendritic cells, as well as memory and senescent T-cells. These cytokines induce expansion of OCPs which in turn may contribute to the maintenance of inflammation through their capability to produce proinflammatory cytokines themselves and recruit other inflammatory cells, rendering the inflammation chronic. Osteoclastogenesis and inflammation are directly proportional to OCP levels in the peripheral blood [95]. Characteristic of an aged immune profile is the accumulation of activated memory cells expressing RANKL, preferentially resident in the bone and secreting osteoclastogenic proinflammatory cytokines. Therefore, through inflammation and its mediators the immune system

influences not only the immunological defense reactions, but each organ in the body, including bone.

The immunophenotypical analysis of peripheral blood lymphocyte subsets confirms the deep involvement of the immune system in bone remodelling. CD3+ T-lymphocytes are increased in osteoporotic patients, as well as their CD4+/CD8+ ratio [55, 93], whereas CD20+ B lymphocytes are significantly decreased. Moreover, an expansion of the CD8+CD56+ lymphoid subset has been described [54]. These are killer/effector lymphocytes producing large amounts of the inflammatory cytokine TNF-α. Finally, in osteoporotic patients there is an increase in CD45RO+ memory lymphocytes, whereas the CD45RA+ naive subset is markedly decreased [31]. Based upon their homing characteristics, cytokine production, and effector functions, memory T-cells have been further subdivided into central memory and effector memory T-cells [38, 62, 79, 103]. These subsets are identified by the presence and absence of a set of cell surface markers. $CD8^+$ effector memory T-cells are further subdivided into 2 subsets, T-effector memory CD45RA negative and T-effector memory CD45RA positive, whereas CD4+effector memory cells are primarily CD45RA negative and only few cells are CD45RA positive; however they are increased in ageing [48]. During ageing the number of central memory CD8+ T-cells is significantly reduced, whereas the number of effector memory CD45RA positive CD8+ T-cells is increased [47, 100]. These memory cells are mainly senescent and proinflammatory cells, able to secrete large amounts of proinflammatory cytokines involved in the regulation of bone turnover. These findings are particularly interesting if we consider that the same immune profile (accumulation of activated cells and memory/effector lymphocytes secreting proinflammatory cytokines) characterizes not only immunosenescence, but also other peculiar immunological conditions notably associated with osteoporosis, such as chronic viral infections, AIDS, rheumatoid arthritis, etc. Another important mechanism which could link inflammageing and osteoporosis is the regulation of immune functions by T-regulatory cells (Tregs). The role of intrathymically generated CD4+CD25+ regulatory T-cells in the control of allergy and asthma is well known [3]. Antiinflammatory, antiproliferative and antiautoreactivity Tregs express innate immunity receptors and respond to proinflammatory signals and products of inflammation. Such natural regulation of Treg by immune responses to nonself may well explain the alarming epidemiology of allergic and autoimmune diseases in wealthy societies, where a variety of childhood infections have become rare or absent [19, 27]. Suppression through natural or professional CD4+CD25+ Tregs is primary cell-contact-dependent but is subsequently followed by cell-contact-indipendent T-cell inhibition mediated by second-generation T-regulatory cells (Tr1 and TH3) via the soluble factors IL-10 and TGF-β [79, 106]. Both these cytokines are able to antagonize immune mediated bone resorption. Thymic dysfunction which accompanies ageing could compromise Treg generation and maturation, facilitating inflammatory processes and osteoporosis. Some authors described an increase in CD4+CD25high regulatory T-cells during ageing [49, 115], which however are quite dysfunctional. Suppressive activity of Treg cells declines with age [116] probably because of age-dependent thymic atrophy or the senescent peripheral environment. Mature and activated dendritic

cells, characteristic of the senescent immune profile, produce proinflammatory cytokines, including IL-6, which render responder T-cells refractory to the suppressive effect of Tregs [61].

5 Regulatory Immune Mechanisms: The Cytokine Network

Changes in the cytokine milieu are major characteristics of ageing process as well as of age-related diseases [17]. The remodelling of the cytokine network is the hallmark of inflamm-ageing [36]. There is a complex network linking the different cytokines involved in immune mediated bone remodelling. Lymphocyte activation does not always lead to osteoporosis, the final result depending on the specific cytokines produced and their reciprocal interactions [130]. There are stimulators and inhibitors of bone resorption. These factors may elicit their effects directly, by acting on the osteoclast precursor or mature cells, such as RANKL, TNF-α, IL-1 and prostaglandin E2 (PGE2), or indirectly, via another cell type, in most cases to modulate RANKL/OPG expression [7, 16, 21, 63, 70], for example parathyroid hormone-related peptide (PTHrP), PGE2, IL-11, IL-17 [7, 21, 70].

For example, TNF-α has the potential to regulate osteoclast differentiation and function in a number of ways. It may promote osteoclastogenesis indirectly through the induction of the expression of RANKL and colony-stimulating factor-1 (CSF-1) in bone-marrow stromal cells and bone-lining cells [7]. Alternatively TNF-α may act directly on the osteoclast precursors to promote osteoclast differentiation. TNF-α may function to increase the CD11b+ osteoclast precursor cell population [60].

The pro-inflammatory cytokine IL-1 signals through its receptor IL-1R1. This interaction is inhibited by the presence of the soluble antagonist IL-1Ra, which competes with IL-1 for binding to the IL-1R1. In the presence of CSF-1, IL-1 can act directly to promote the fusion of mononuclear osteoclast precursors to form osteoclasts [65] and can promote the survival and function of mature osteoclasts. Like TNF-α, the capacity of IL-1 to promote immune response in inflammatory arthritis has made IL-1 a target for therapeutic blockade. The approved therapeutic agent for blockade IL-1 signaling in RA is a recombinant form of IL-1Ra (anakinra) [28, 48, 52, 65, 129, 130], and use of anakinra has proved efficacious in the treatment of inflammatory arthritis with retardation of focal bone erosion in a significant number of patients.

IL-4 is one of the inhibitor cytokines. Moreover, its hyperproduction characterizes an atopic background and stimulates IgE synthesis. Interestingly, in some cases, allergy and TH2 immune profile could result protective towards osteoporosis and other inflammatory diseases [32]. A TH2-mediated atopic disease protection in TH1-mediated diseases such as RA has been described [78]. In an unpublished study, bone mineral density in allergic patients who have not undergone cortisone therapy resulted higher compared to sex and age-matched healthy controls. An

inverse correlation between bone mineral density and total IgE, that are markers of atopy, also exists.

IL-6 is a key regulator of osteoclast differentiation in response to estrogen deficiency in postmenopausal bone loss. IL-6 is significantly increased during ageing and its level strongly correlates with the risk of osteoporotic fractures [78].The pleiotrophic proinflammatory cytokine IL-6 has been detected at elevated levels in synovial fluid and sera of RA patients with active disease. In addition IL-6 and its soluble receptor (sIL-6R) levels in RA patients have been correlated with the degree of radiographic damage.

IL-7 is a cytokine that stimulates thymic T-cell production and induces the expansion, activation, and differentiation of mature circulating T-cells. IL-7 induces bone loss in vivo, presumably by stimulating the differentiation of osteoclast precursor cells into osteoclasts. IL-7 also upregulates RANKL production in T-cells. IL-7 induces proinflammatory and osteoclastogenic cytokine production and the expansion of B220+ IgM- B cell precursors. These cells could lead to bone destruction by overexpressing RANKL or, alternatively, by differentiating into OCPs in response to M-CSF and/or RANKL.

IL-11 regulates the growth and development of hematopoietic stem cells. Like IL-6, IL11 has been implicated in mediating osteoclast differentiation through the upregulation of RANKL expression in cells of the osteoblast lineage.

IL-17 is a proinflammatoy cytokine secreted predominantly by activated CD4+CD45RO+ memory T-cells [123]. Through its ubiquitously expressed receptor, IL-17R, leads to the activation of the adapter molecule TNF receptor associated factor 6 (TRAF6) and subsequent modulation of target gene expression via signalling through the NF-kB and mitogen activated protein tyrosine kinase pathways [112, 73]. IL-17 induces the production and secretion of IL-1, IL-6, IL-8, TNF-α, GM-CSF and PGE2. IL-17 also induces the expression of RANKL and decreases OPG expression in both RA synoviocytes and cells of the osteoblast lineage [111].

IL-18, a member of the IL-1 superfamily of cytokines, is present at elevated levels in the synovial membrane, synovial fluid, and serum of RA patients. Originally, IL-18 was demonstrated in vivo to inhibit osteoclast differentiation indirectly via the induction of GM-CSF expression by both cells of the osteoblast lineage and activated T-cells. IL-18 may promote osteoclast differentiation by inducing T-cell expression of RANKL.

Osteopontin, also known as Eta-1 (early T-lymphocyte activation gene-1), is a secreted phosphorylated glycoprotein that functions both in inflammation and bone remodelling. Important in mediating T-helper 1 cell immune responses, osteopontin is produced by activated T-cells and macrophages. It interacts with CD44 and integrin receptors to promote chemotaxis and migration of monocyte-macrophage cells and enhances B-cell proliferation and antibody secretion. It is also produced by both osteoclasts and cells of the osteoblast lineage and acts to promote cell-matrix adhesion via integrin [107]. Interplay between interferon and other cytokine systems in bone metabolism.

TRAF6 is a crucial signalling molecule regulating a diverse array of physiological processes, including adaptive immunity, innate immunity, bone metabolism and the development of several tissues including lymph nodes, mammary glands, skin and the central nervous system. It is a member of a group of six closely related TRAF proteins, which serve as adapter molecules, coupling the TNF receptor (TNFR) superfamily to intracellular signalling events. Among the TRAF proteins, TRAF6 is unique in that, in addition to mediating TNFR family signalling, it is also essential for signalling downstream of an unrelated family of receptors, the IL-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily. TRAF6 therefore represents an important target in the regulation of many disease processes, including immunity, inflammation and osteoporosis [131]. There exists an intimate interplay between the bone and the immune system. Skeletal bone is more than a frame on which to hang flesh and organs, it is also the source of bone-marrow-derived haematopoietic cells. Many myeloid lineage haematopoietic cells express receptors such as CD40, RANK and TLRs, which use TRAF6 for signalling and are involved in the generation of adaptive and innate immunity. Interferon (INF)-γ interferes with the osteoclast differentiation induced by RANKL, and this mechanism is critical for the suppression of pathological bone resorption associated with inflammation.

Also antigen presenting cells (APC), in addition to stimulate bone resorption, could negatively regulate osteoclastogenesis through up-regulation of the RANKL decoy receptor OPG. The secretion of IFN-γ, in particular, appears to be crucial and multifaceted in immune mediated osteoclastogenesis by shifting myeloid stem cell differentiation from OCPs to dendritic cells. During senescence there is an impaired OPG production as well as an impaired IFN-γ production [4, 53], contributing to a derangement of the global counter-regulatory system.

Activated T-cells exert both positive and negative control on osteoclastogenesis. In fact, in addition to the osteoclast activator RANKL, they express IFN-γ too, which binds to its receptor on osteoclasts. This induces the proteasomal degradation of transcription factor TRAF6 leading to an inhibition of the signal transduced by RANKL and an inhibition of osteoclast function [113]. This direct inhibitory effect of IFN-γ on osteoclasts contrasts with its indirect stimulatory activity through lymphocyte activation and cytokine production. In fact IFN-γ is also a potent inducer of expression of Class II histocompatibility complex antigens on antigen presenting cells. This increases T-cell stimulation mediated by antigen receptor, inducing further immune activation, proinflammatory cytokine production and consequent osteoclast stimulation.

RANKL induces the INF-β gene in osteoclast precursor cells, and this induction constitutes a critical aspect of the negative feedback regulation mechanisms of RANKL signalling to suppress excessive osteoclastogenesis. An important function of signal transducer and activator of transcription I (Stat 1), the essential transcription factor for both type I and type II IFN responses, is therefore the regulation of osteoblast differentiation.

The binding of RANKL to its receptor RANK results in the recruitment of TRAF6, which activates NF-kB and c-jun N-terminal Kinase (JNK) pathways and induces c-Fos expression. The effect of T-cells on osteoclastogenesis therefore depends on the

Table 1 Cytokines involved in bone remodelling

Osteoclastogenesis Stimulators	Osteoclastogenesis Inhibitors
TNF-α	IL-4
IL-1	IL-10
IL-6	IL-13
IL-7	IL-18
IL-11	GM-CSF
IL-15	TGF-β
IL-17	IFN-γ

balance between RANKL and IFN-γ. IFN-γ signals the cell through activation of the transcription factor signal transducer and activator of transcription 1 (Stat 1). The active form of Stat 1, termed IFN-γ activated factor (GAF), induces target genes of IFN-γ either directly or through the induction of the transcription factor IFN regulatory factor-1 (IRF-1 [53]). During acute immune reaction, an enhanced production of IFN–γ counterbalances the augmentation of RANKL expression and reduces aberrant osteoclast formation.

6 Osteoporosis and Immune Mediated Diseases

Rheumatoid arthritis, seronegative spondyloarthropathies including psoriatic arthritis [132], and systemic lupus erythematosus (SLE) are all examples of rheumatic diseases in which inflammation is associated with skeletal pathology [20, 71, 120, 121]. Although some of the mechanisms of skeletal remodelling are shared among these diseases, each disease has a unique impact on articular bone or on the axial or appendicular skeleton. RA is the prototype for an inflammatory arthritis, in which inflammation is associated with progressive bone resorption. Several immunological findings are shared by RA and senescence, suggesting similar immunopathogenetic mechanisms for bone resorption in both these conditions [43]. Patients with RA have age-inappropriate telomeric shortening of haematopoietic precursor cells. Their output of novel T-cells from the thymus is impaired. The peripheral T-cell pool is occupied by functionally altered T-cells, which bear the characteristics of prematurely aged lymphocytes. Global T-cell defects include a sharp contraction in T-cell diversity, the accumulation of expanded clonotypes and preponderance for senescent T-cells in the T-cell compartment [42, 128]. This immune phenotype is shared by other pathologic conditions characterized by increased incidence of osteoporosis, such as HIV infection. The overproduction of proinflammatory cytokines, such as TNF-α, further impairs the function of haematopoietic stem cells, aggravating the impact of a genetically determined risk factor. Recently, a new disease model for RA has been proposed [128]. Instead of restricting the biological role of HLA-DRB1 molecules to the presentation of arthritogenic antigens, these HLA

molecules or genes in linkage disequilibrium to the B1 locus, could regulate haemopoietic stem cell biology. In HLA-DR4+ individuals, stem cells proliferate excessively, giving rise to prematurely aged T-cells. If combined with additional restrictions in thymic T-cell production, the T-cell pool becomes senescent, with restriction in diversity and limited ability for clonal burst. The same phenomenon during senescence is triggered by lifelong antigenic burden and thymic atrophy [40]. Senescent T-cells express novel regulatory receptors, are proinflammatory and are prone to autoreactivity, promoting chronic inflammatory lesions, such as rheumatoid synovitis and osteoporosis [127, 128].Three major forms of bone loss have been described in RA: focal articular erosions, a hallmark of RA; periarticular bone loss, occurring adjacent to inflammed joints; and generalized osteoporosis, leading to an increase in fracture risk. The synovium is the major target of the inflammatory process in RA. Activated lymphocytes in the inflammed synovium overexpress RANKL and TNF-α which stimulate bone-marrow osteoclast progenitors to proliferate and enter the blood stream. In turn, activated macrophages in inflammed joints produce various chemokines, small inflammatory chemotactic cytokines, which drive osteoclast progenitor migration and homing in the periarticular bone. The elevated concentration of RANKL and TNF-α in the rheumatoid synovial fluid stimulates maturation and activation of osteoclasts which resorb bone. Circulating OCPs secrete inflammatory cytokines amplifying inflammatory circuits at a systemic level [126]. Circulating osteoclast precursor number has been proposed as a marker of osteoporotic risk and therapeutic response.

TNF-α also induces osteoblast apoptosis, decreasing bone formation. Histologic examination of the periarticular osteoporotic region in patients with RA shows functional Fas expression and apoptosis in osteoblasts. IL-1β and TNF-α regulate osteoblast cell number by up-regulating the Fas-mediated apoptosis of osteoblasts [117]. The defective clearance of apoptotic cells is associated with autoimmunity and inflammation [101]. Under normal conditions, clearance of apoptotic cells by phagocytic cells is associated with secretion of antiinflammatory cytokines, including IL-10 and TGF-β1, resulting in the inhibition of inflammation. However, under pathological conditions associated with excessive apoptosis and/or decreased clearance of apoptotic cells, apoptotic cells may directly induce caspase-1 dependent secretion of IL-1β and IL-8 or under secondary necrosis may induce secretion of other proinflammatory cytokines. During ageing a defective clearance of apoptotic cells as a result of poor phagocytosis by aged dendritic cells results in secondary necrosis and release of endogenous ligands for toll like receptors to activate phagocytic cells to differentiate into more mature phenotype and secrete proinflammatory cytokines (e.g. TNF-α and IL-6) [47]. In immune mediated osteoporosis, in addition to systemic overproduction of bone-resorbing proinflammatory cytokines, nitric oxide and prostaglandin also play a role, mainly stimulating osteoblast apoptosis.

All these basic immunological mechanisms have important clinical implications. In RA and psoriatic arthritis the degree of inflammation and disease activity correlate with focal erosions and systemic osteoporosis. Bisphosphonates are drugs widely used in the therapy of osteoporosis, able to improve BMD and decrease the

risk of fractures in patients with RA and steroid-induced osteoporosis [78]. They are able to regulate cell growth and apoptosis and may inhibit the inflammatory response of macrophages. They exert antiinflammatory activity by the inhibition of the release of inflammatory mediators from activated macrophages, such as IL-1, IL-6 and TNF-α and prevent dexamethasone-induced growth retardation and apoptosis both in osteoblasts and chondrocytes [99, 119, 121].

Blockade of the RANKL/RANK signalling pathway represents an attractive target for therapeutic intervention in the prevention of bone loss in RA. In initial human trials, the effects of OPG.Fc were examined in a cohort of postmenopausal females [14]. A single injection of OPG.Fc resulted in a sustained reduction in the level of urinary N-telopeptide, a stable collagen breackdown product, consistent with a reduction in bone resorption activity. However, the essential role of the RANKL/RANK/OPG pathway in physiological bone remodelling would suggest that modulation, rather than complete inhibition, of this pathway may be the desirable aim of therapeutic intervention. The development of small molecules (or peptidomimetics) that target the RANKL/RANK signalling pathway (molecules that mimic OPG action or modulate endogenous OPG mRNA expression [24, 121]) may provide a greater ability to modulate inflammation-induced osteoclast differentiation without complete inhibition of this pathway. Blockade TNF-α activity using biologic agents, including recombinant soluble p75TNFR (etanercept), a chimeric mouse-human anti-TNF-α antibody (infliximab), and a fully humanized anti-TNF-α antibody (adalimumab), has demonstrated efficacy in reducing the clinical signs and symptoms of RA and in retardating radiographic progression of focal bone erosions [95, 121](78-87 di Walsh). Blockade of TNF-a signalling by infliximab treatment or RANKL signalling by OPG.Fc treatment results in decreased osteoclast cell numbers and subsequently reduced bone erosions.

Since RANKL is expressed on activated T-cells, and is crucial for T-cell-dendritic cell communication, one might expect massive bone resorption under most inflammatory conditions. Although RANKL-expressing T-cells in chronic inflammatory conditions such as RA and inflamm-ageing, can stimulate osteoclasts leading to bone destruction, the constant activity of T-cells fighting the universe of antigens to which we are exposed does not usually cause extensive bone loss. As previously exposed, a crucial counter-regulatory mechanism whereby activated T-cells can inhibit RANKL mediated osteoclast development and activation is through the action of IFN-γ. In mice deficient for the IFN-γ receptor, bone destruction in an autoimmune arthritis model is greatly exacerbated. While T-cells involved in inflammatory responses express RANKL, they also secrete IFN-γ. IFN-γ can block RANKL-mediated osteoclastogenesis, possibly through the activation of the ubiquitin-proteasome pathway leading to TRAF6 degradation [131]. Given the essential roles of TRAF6 in immunity and a diverse array of biological processes, it is desirable to obtain TRAF6 inhibitors to facilitate the development of therapeutics for controlling inflammation and a wide range of diseases, such as osteoporosis and other osteolytic conditions[107].

Interestingly, despite T-cell infiltration observed in arthritic joints, IFN-γ expression in these T-cells is suppressed. The paucity of IFN-γ and the enhanced expres-

sion of RANKL may underlie the activation of osteoclastogenesis in arthritis. T-cells which infiltrate rheumatoid synovium have an expression of surface markers for memory T-cells, a low production of IFN-γ or IL-2, and hyporesponsiveness to in vitro restimulation.

Therefore, not always immune activation exerts resorptive effects on bone, probably explaining the different clinical manifestations of certain immune diseases. For example, only 4–6% of patients with SLE develop erosive arthritis, despite the frequent articular involvement on presentation (50%). The hypothesis to explain this phenomenon is that systemic interferon-α diverts the bone-marrow-derived myeloid precursors away from the osteoclast lineage and stimulates their differentiation into dendritic cells. In SLE patients there is an increased interferon production and anti-TNF-α therapy is scarcely effective (39). Therefore, the innate immune TNF/IFN axis in patients with autoimmune disease dictates their erosive phenotype.

Although it is well documented that IFN-γ has a bone-protective effect in antigen-specific autoimmune arthritis, recent studies suggest that IFN-γ may have a causal role in the bone loss associated with estrogen deficiency. Pacifici et al. propose that IFN-γ activates antigen presentation through Class II transactivator (CIITA) induction, leading to the accumulation of a TNF-α-producing T-cell populatio[121].

7 Immune-mediated Postmenopausal Osteoporosis

There is progressive loss of bone tissue after natural or surgical menopause, leading to increased fractures within 15–20 yr from the cessation of ovarian function [88]. Postmenopausal osteoporosis should be regarded as a product of an inflammatory disease triggered by estrogen deficiency. Osteoblast, osteocytes, and osteoclasts express functional estrogen receptors. These receptors are also expressed in bone-marrow stromal cells, the precursors of osteoblasts, which provide physical support for nascent osteoclasts, T-cells and B-cells. Estrogen signals through 2 receptors, ERα and Erβ. Bone cells contain both receptors.

Although estrogen is established to have direct effects on bone cells, recent studies have identified additional unexpected regulatory effects of estrogen centered at the level of the adaptive immune response [125]. Estrogens have important roles in the regulation of immune function. Ovariectomy increases the number of TNF-producing T-cells. Estrogen deficiency results in a marked increase in proinflammatory cytokines, including IL-1, IL-6, TNF-α, M-CSF, IFN-γ and others. Estrogen deficiency is also associated with decreased production of OPG and TGF-β, which counteract bone resorption. TGF-β is a powerful repressor of T-cell activation. Estrogen deficiency upregulates IFN-γ production through TGF-β downregulation. Generally, following an innate immune activation, IFN-γ functions as an antiresorptive agent. Conversely, when T-cell activation occurs through an adaptive immune response, as in estrogen deficiency, IFN-γ stimulates bone resorption. Estrogens also repress the production of IL-7, a potent stimulators of T and B proliferation and inducer of bone destruction [125].

Reactive oxygen species (ROS) may play a role in postmenopausal bone loss by generating a more oxidized bone microenviroment. The NO donor nitroglycerin is also reported to prevent bone loss in ovariectomized rats. OCs have been shown to both generate and be activated by ROS. Glutathione peroxidase, responsible for intracellular degradation of hydrogen peroxide, is the predominant antioxidant enzyme expressed by OCs and is upregulated by estrogen. ROS are important stimulators of antigen presentation by dendritic cell (DC)-induced T cell activation. Antioxidants potently inhibit DC differentiation and activation of T-cells in part by suppressing expression of MHC Class II and costimulatory molecules in response to antigen. ROS are also generated upon DC interaction with T-cells and can reduce T-cell lifespan by stimulating T-cell apoptosis. Estrogen deficiency lowers antioxidant levels, thereby increasing ROS. Additionally, estrogen deficiency augments TNF expression by enhancing OC-mediated TNF production and by stimulating APC-induced expansion of the TNF-producing T-cells that are central to bone destruction [68].

There are 2 transcription factors, NF-kB and AP-1, which are regulated by estrogen and control the expression of IL-12 and IL-18 in macrophages. The stimulation of INF-γ secretion through the enhanced production of INF-γ-inducing cytokines IL-12 and IL-18 by macrophages is another meccanism by which estrogen deficiency activates immune system [23, 37, 91].

In summary, menopause increases T-cell activation and proliferation by increasing APC activity of macrophages through increased MHCII expression and by reducing T-cell apoptosis. These actions result in the expansion of the pool of activated T-cells in the bone-marrow which are responsible for the chronic stimulation of osteoclast formation and consequent bone loss. [52, 59, 84, 85, 91, 114]

8 The Immune Genetic Background of Osteoporosis

Osteoporosis could be considered an inflammatory disorder with a strong genetic component. Bone mineral density is largely controlled by genetics. Proinflammatory cytokine polymorphisms are genetic markers of both inflamm-ageing and osteoporosis. The genetic background which favours the onset and progression of osteoporosis is the same that determines strong inflammatory immune responses through the hyperproduction of inflammatory cytokines and/or the decreased secretion of antiinflammatory and regulatory factors. A number of cytokine genes and genes involved in inflammatory responses are polymorphic and may be important for defining the magnitude of the individual responses to a given environmental stimulus of cytokine production. The list includes genes that affect cytokine expression, binding of cytokines to their receptors, genes involved in the cytokine signalling pathways, and many others. There is a growing number of studies that have examined the effects of these cytokine polymorphisms on postmenopausal bone loss. Tsukamoto et al. investigated an association between a CA-repeat polymorphism at the IL-6 gene locus and BMD of radial bone in 472 postmenopausal

Japanese women. The 73 women who possessed an A1 allele (134 bp, containing 18 repeats of CA) had significantly lower BMD than those who did not carry an allele of that size. Keen et al. examined the relationship between annual rates of change in BMD and an 86-bp variable number tandem-repeat polymorphism of the IL-1ra gene in108 women without hormonal replacement therapy within 5yr of menopause. They observed that carriage of at least one copy of the A2 allele was associated with reduced bone loss at the spine. Lagdahl et al. also showed that genotypes associated with a low IL-1ra production (A1A1/A3) were significantly more frequent in women with osteoporotic fractures compared with normal individuals, but this polymorphism had no effect on bone loss in another study of 487 postmenopausal Danish women. IL-6 polymorphisms are able to influence the risk of osteoporosis as well as other chronic disorders involving IL-6 activity [34]. Two promoter polymorphisms regulating IL-6 gene expression, -572 and -174 G>C, are associated with circulating levels of C-reactive protein and markers of bone resorption in postmenopausal women.For example, a single nucleotide polymorphism in the promoter region of the IL-6 gene at position -174 (G>C) has been reported to be associated with a variety of major age-related diseases which share an inflammatory background, as well as with osteoporosis (EVOS study). Individuals with the G genotype have significantly higher plasma IL-6 values than do individuals with the C genotype. Therefore the -174 G>C single-nucleotide polymorphism in the promoter region of the IL-6 gene is functional in vivo with an increased inflammatory response associated with the G allele [15]. Considering the central role of IL-6 in bone resorption, this finding could have clinical relevance.

A relationship between the production of IL-1 and IL-6 by whole blood cells, bone mineral density and polymorphisms in IL-1 system and IL-6 gene in postmenopausal women has also been documented [8, 66]. The loci for the human IL-1α, IL-1β and Il-1Ra are all linked within the proximal region of the long arm of chromosome 2. IL-1β and IL-1Ra are involved in high turnover bone loss after menopause [25]. Different polymorphisms have been described in the IL-1β gene and at least 2 of them could influence protein production: one is located within the promoter region, the other in exon 5 [24, 25, 86]. Polymorphisms in the IL-1β exon 5 may influence gene transcription and protein production [86]. The Taq I IL-1β exon 5 gene polymorphism is one of the candidate genetic markers responsible for osteoporosis in postmenopausal women, and this genetic locus may play a central role in postmenopausal trabecular bone loss [25]. Five alleles of the IL-1Ra gene have been reported, corresponding to 2, 3, 4, 5, and 6 copies of an 86-basepair sequence repeat located in intron 2 [110]. Bone metabolism as well as inflammatory processes are influenced by the vitamin D receptor gene (VDR). The VDR gene may be involved in BMD differences, bone metabolism and inflammatory processes in ankylosing spondylitis [83].

With respect to TGF-β, a 1-base delection in intron 4 of the TGF-β1 gene has been associated with low BMD, increased bone turnover, and an increased rate of fragility fractures in osteoporotic Danish and Italian women [5].

9 Conclusion

What is the finality of the close relationship between inflammation and bone remodelling? One possible explanation could be that bone has not only structural, but also storage function for calcium and phosphate salts and defense functions. Postmenopausal osteoporosis should be regarded as the product of an inflammatory disease bearing many characteristics of an organ limited autoimmune disorder, triggerd by estrogen deficiency and brought about by chronic mild decreases in T-cell tolerance. Why such a pathway should have emerged is intriguing. One explanation is suggested by the need to stimulate bone resorption in the immediate postpartum period in order to meet the markedly increased maternal demand for calcium brought about by milk production. The signal for this event is the drop in estrogen levels early in the postpartum. Postmenopausal bone loss should be regarded as an unintended recapitulation of this phenomenon. Another response to delively is the restoration of normal immune reactivity and the loss of tolerance to the fetus. It is tempting to speculate that cessation of ovarian function induces bone loss through an adaptive immune response because natural selection has centralized these 2 key adaptations to postpartum within the immune system [107, 125].

Inflammatory responses require a ready supply of calcium for cellular activation and signal transmission. Also in this case, as well as in lactation during the postpartum, calcium derives from bone resorption. During evolution, T-lymphocyte assumed the central role of director of these complex integrated systems. In this perspective, osteoporosis may reflect a state of disequilibrium between structural demand for calcium and phosphate and their biological demand during metabolically active states such as inflammation [135]. Therefore inflammation could be considered the main force driving osteoporosis.

The correct understanding of the complex language existing between immune system and bone during ageing is the essential requirement for the individualization of new and effective therapeutic targets for both osteoporosis and inflammation.

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Part V
Modulation- Nutrition

Protein-Energy Malnutrition as a Determinant for Immuno-Senescence

Anis Larbi, Bruno Lesourd and Tamas Fulop

Contents

Abstract: Human aging is associated with a loss of function involving organs or systems leading to pathologies such as cognitive impairment, macular degeneration, sarcopenia, frailty, cancer and increased susceptibility to infections. The erosion of the immune system is one of the age-associated failures observed. The delayed time for recovery and the increased susceptibility to infections with aging are directly associated with immune dysfunctions. Moreover, interventions aiming to protect the aged population such as vaccination have a limited efficiency. The eroded innate and adaptive immunity are responsible for this phenomenon. Several prophylactic and therapeutic approaches could restore immune function of immuno-depressed individuals. The nutritional approach is suitable for the aged-population since it requires less care than any medical approach and its cost is much lower which is an important factor when considering the health burden costs. Nevertheless, it is necessary to carefully and critically analyze the recent development in this field. In

A. Larbi (✉)
Center for Medical Research
Section for Transplant-Immunology and Immuno-Hematology
Tuebingen Aging and Tumor Immunology group
University of Tuebingen
Centre for Medical Research
Waldhornlestr. 22. 72072 Tuebingen, Germany
E-mail: anis.larbi@medizin.uni-tuebingen.de

B. Lesourd
EA 2431, Faculté de Médecine, 28 Place Henri Dunant
63001 Clermont-Ferrand and Hôpital Nord du CHU de Clermont-Ferrand
BP 36, 63118 Cebazat, France

T. Fulop
Centre de recherche sur le vieillissement
université de Sherbrooke
1036 rue Belvédère Sud
J1H 4C4, Sherbrooke, Qc, Canada

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this review, we will first discuss the age-associated changes in immune functions, collectively named immune-senescence then discuss the age-associated changes in nutritional intake and metabolism with a particular interest in protein-energy malnutrition (PEM) to finally outline some of the candidate interventions to protect against PEM and immuno-senescence.

1 Introduction

The reason why we age is not known however several hypotheses were put forward to explain the aging process as well as inter-individual inequalities towards aging. Thus, some intrinsic factors such as the genetic background and some extrinsic factors such as the socio-economic status or nutritional were shown to influence the aging process. Aging is associated with a variety of changes and adaptations. Alterations in brain, immune, cardiovascular as well as other metabolic functions were shown in the aged individuals (Cutler et al. 2006; El Sohl et al. 2006; Rosano et al. 2006). One characteristic of aging is the significant increased susceptibility to infectious diseases such as tuberculosis. Aged individuals significantly need a longer period to recover from an infection compared to young individuals (Weng 2006). Nowadays in developed countries, with the increased medical care, socio-economical status and recent progresses in medicine, the aged population is defined as over 65 year, of age. Within the aged-population, several classes can be distinguished, i.e., young old (65–75 years old), old old (75–95), and very old (over 100 years old). While the major cause of death for middle-aged and young old individuals is cancer, the main diseases occurring and responsible for death over the age of 75 are infections. This shows that the forthcoming increase frequencies of over-75 individuals in developing countries, concomitantly to their increase in developed countries will need much more attention. This means that a better knowledge of age-associated immune dysfunctions is needed to be able to propose prophylactic or therapeutic treatments. Among the immune dysfunctions known so far it is well-accepted that T-cell functions are the most eroded with age (Fulop et al. 2005).

2 T-cells and the Immune Response

The immune responses encompass the non-clonotypic innate and the clonotypic adaptive immunities. Cells from the innate immune system, such as neutrophils, will respond in a nonspecific manner to antigens (Solana et al. 2006) while cells from the adaptive immune system such as T-cells will be activated in a specific manner. This is because of the presentation of the epitope derived from the antigen by the major histocompatibilty complex expressed on the surface antigen presenting cells (APC) and recognized by the T-cell receptor which is also called signal 1. CD4+ T-cells will recognize epitopes presented on class I MHC while CD8+ T-cells recognize epitopes presented on class II MHC. Other co-stimulatory molecules

expressed on T-cells, such as CD28, will be ligated: this stands for signal 2, and allows further activation of the cell (Nel 2002). Following activation, T-cells will secrete factors and proliferate. One of these factors, interleukin-2 (IL-2) will lead the clonal expansion of the responding cells. The autocrine and paracrine effect of IL-2 is considered as signal 3. Altogether, signals 1-3 will drive the induction, duration and termination of the immune response. Other cytokines will drive the type of the response. At the end of the response, most of the cells will die by a programmed cell death, Known as activation-induced cell death. Only a minority of cells will survive and belong to the memory population. Memory cells will be circulating and will serve for immuno-surveillance. Another encounter with the same antigen will lead to a faster and more intense response. Depending on the ability of the immune system to respond firmly to pathogen aggression, the individual will have a fast or delayed recovery.

3 Immune-Senescence

Immune-senescence was first characterized by a reduced T-cell proliferative capacity as well as reduced ability to produce IL-2 following stimulation (Pawelec 2003). These functional changes are accompanied and somehow explained by changes in the phenotype of these cells (Weng 2006). One of the best examples is the significant increase of the CD28-negative populations with aging, mostly within the CD8+ T-cells (Boucher et al. 1998). Several hypotheses were put forward to explain this phenomenon. Among these, we think that cytomegalovirus (CMV) infection which is an asymptomatic but chronic infection, is responsible for the expansion of CMV-specific cells. These cells belong to the effector memory CD8+ subsets with a CCR7-CD45RA+/-CD27+/-CD28-phenotpye. The reason for the expansion of CMV-specific cells is not known and it is unclear whether the immune system needs it to control CMV or if this is evidence that CMV is taking advantage over the immune system. Anyhow, this expansion is filling the immunological space and leaves less space for T-cells specific for other antigens (Pawelec et al. 2005). This combined with thymic atrophy could be responsible for the decreased frequency of naïve CD8+ T-cells with aging and also in CMV-seropositive young individuals. Other hypothesis, i.e., the alteration in signal transduction from the membrane to the nucleus could explain why T-cells from old individuals were less able to proliferate (Fulop 1994). Many studies showed that several pathways of T-cell receptor signaling cascade following TCR/CD28 triggering are altered (Pawelec et al. 2001). More recently, we have shown that a common alteration might be responsible for the overall alterations, i.e., membrane rafts (Larbi et al. 2004). Membrane rafts are motile domains of the membrane enriched in certain class of lipids such as cholesterol, sphingomyelin and saturated fatty acids. Their composition and structure is very peculiar but necessary for the signalosome formation, which includes the molecules necessary to reach the full-state of cellular activation (Nunes et al. 2006). Thus, one has to be aware that every change in the lipid environment will induce

some changes in the membrane lipid and protein composition which in turn influences T-cell responsiveness (Garcia et al. 2001). Altogether, these alterations might explain why the aged individual is more susceptible to infections and why recovery is longer.

4 Immune-Senescence and Nutrition

There is no need to discuss the fact that aged individuals have different nutritional needs as well as intake compared to young individuals (Hays et al. 2006). The quantitative and qualitative levels of calories needed become different with aging. Nevertheless, it is important to provide a sufficient dietary intake to cover the essential needs to ensure a proper functioning of the immune system (Chandra 2003; Lesourd 2006). Several studies demonstrated that aged individuals lack of certain essential elements (Lesourd 2004). Several dietary deficiencies such as zinc, selenium, beta-carotene, vitamin B6, B12, C, D, E, and folic acid were demonstrated (Flynn et al. 2003; Vaquero 2002). Such a lack would have a detrimental acute or chronic effect on immune functions (Ritz al. 2006; Chandra 2002). Aged individuals display an eroded immune system and are most of the time deficient for many essential elements. These individuals may be at risk of infectious diseases and those with prolonged malnutrition can display signs of chronic inflammation (Chung et al. 2006). The resulting chronic inflammation, which is also occurring in certain pathological cases, will demand a significant amount of energy, which is not always available. (Ahluwalia 2004). Therefore, it is common for energy sources which are already expended to be solicited again (Richardson et al. 2003), increasing susceptibility to other diseases (Stenvinkel 2001). These deficiencies have been associated to several immune functions (Chandra 2004; Johnson et al. 1992).

It is easy to assess the circulating level of the essential elements and to detect any disequilibrium between the intake and the amount used up. However, the metabolism of all the nutritional elements is not fully known despite their importance in several processes. This is the case for lipids. Apart from the known increase in circulating cholesterol levels with age and its role in the onset and development of cardiovascular diseases, very few studies have shown the changes in level of other classes of lipids with age. In vitro studies clearly demonstrated that changing the lipid composition of culture media modulates immune cell functions, especially those of T-cells (High et al. 2003; Ponnappan et al. 1996). The role of individual lipids was also analyzed and shown to alter T-cell functions (Calder 2001; Kews et al. 2002). Unsaturated fatty acids were shown to be the most potent T-cell modulators (Zeyda et al. 2003). One consequence of changing lipid compositions is the modification of the plasma membrane lipid composition (Wick et al. 1991). The surrounding lipids will enter the membrane in a passive or active way which both perturb the physico-chemical properties of the membrane. Specialized membrane microdomains called rafts will have a strong influence during lipid exchanges (Zeyda et al. 2002). Signaling molecules associated to membrane rafts will dissoci-

ate when the membrane lipid structure is unbalanced explaining in part the loss of T-cell functions. In the case of T-cells, the majority of membrane raft lipids are saturated, compared to the rest of the membrane. The enrichment of T-cell membranes in polyunsaturated fatty acids will directly inhibit T-cell responses to stimulation (Stulnig et al. 2001). In vivo treatment with a mixture of lipids in humans (Intralipid-20) induced a significantly reduced T-cell proliferative capacity (Larbi et al. 2005) showing a good correlation between in vitro data and the in vivo situation. This clearly shows the critical impact of nutrition in the maintenance of the immune fitness. Since aged individuals display some deficiencies and disequilibrium in dietary intakes, one could easily estimate their effect on the immune system and in the development and maintenance of immune-senescence. More studies are required to demonstrate any pro-inflammatory effect of certain lipids and whether some lipids may have any efficient anti-inflammatory effect, which could be used to reduce the low grade inflammation seen with age and also named inflame-aging. This would require to know the effect of each lipid on cellular functions before suggesting any beneficial effect.

5 Protein-Energy Malnutrition

Under-nutrition occurs when nutrient intake does not balance with nutritional needs. A moderate under-nutrition can later lead to protein energy malnutrition (PEM; Latham 1990). The origin of PEM is in part due to some physiological changes such as decreased smell and taste capacities. PEM is often associated with illness. While more than a third of the institutionalized population displays signs of PEM, less than 5% of the dwelling population shows signs of PEM (Muhlethaler et al. 1995). First signs of PEM are tiredness and a global low potency which can turn into anorexia, weight loss and increased susceptibility to infections as late signs of PEM (Morley 1991). The metabolic changes in PEM include water and electrolytes imbalance, amino acids and proteins deficiencies, carbohydrates and energy deficiencies, hypolipidaemias, hypolipoproteinaemias, hormonal imbalance, deficiency of antioxidant vitamins and enzymes, and decrease in amino acids and trace elements in skin and hair (Brownie 2006). Any illness would increase the level of PEM creating a vicious circle starting with PEM which favors infections which demands much more energy which is not available thereafter decreasing even more the energetic stocks responsible in part for weight loss and frailty (Ambrus et al. 2004). The last stages of PEM are perturbed glucose metabolism, recurrent infection, dehydration, impaired wound healing, and calcium bone loss.

The age-related changes in T-cell subset surface marker expression (CD3, CD45RA) and functions (proliferation) are known to be slimmed when the population studied is very healthy (healthy aging). However, these changes are more important in malnourished elderly and even more in elderly with PEM. Most of immune cell functions including the adaptive and the innate arm are decreased in these individuals diagnosed for PEM. Increasing the food intake can partly reverse

this process unless an inflammatory process is present (Lesourd 2004). Thus, it is important to discriminate between the aging populations. There are different stages of ageing (i) the healthy aging population, which is observed in very healthy elderly individuals who have no nutritional deficit and generally meet the SENIEUR protocol for immuno-gerontologic studies. These individuals display a change in T-cell subset frequencies mainly due to higher number of pathogen recognition over the years but with no change in cellular functions (ii) the unselected aging population, which is observed in most elderly individuals with various micronutrient deficits.

These are still on the safe side because of their capacity to recover some of the immune functions lost due to nutritional deficiencies (iii) the aging population with significant signs of PEM. These individuals have a severely eroded immune system with extended recovery periods which need much energy and use of nutritional reserve directly responsible for the onset of frailty (Lesourd 2006).

6 Nutritional Interventions

Several approaches were tested in order to restore or maintain immune functions. Since nutrition is playing an important role in the maintenance of a functioning immune system, using dietary supplementation is virtually the more interesting because of its cost/feasibility (Bengmark 1998; Heuser et al. 1997). One of the causes of immune-senescence is the oxidative stress (Das et al. 2007) which can chronically damage cell membranes. In order to reduce reactive oxygen species (ROS) one can think about using antioxidants (De la Fuente et al. 2005). So far, very few studies demonstrated a beneficial effect of antioxidant supplementation on immune function of the aged population. Actually, data are still controversial. Some studies showed that some antioxidants can have an inhibitory effect on T-cell functions (Gao et al. 2004; Kolettas et al. 2006; Watson et al. 2005). The explanation for this can be found in the metabolism of these antioxidants when ingested (Aruoma 2003). Scientist must think about the best way to take these agents to be efficiently used-up by the organism and thus see an effect in clinical trials. Also, it is still unclear how and whether antioxidants interact together to provide an efficient protection. Recent work from the Sinclair's group showed the capacity of a red-fruit derived polyphenol named resveratrol (of the polyphenols family) to extent the lifespan of yeasts and worms, with similar studies performed successfully in mice (Baur et al. 2006a; Howitz et al. 2003). Resveratrol is an antioxidant with potent anticancer properties. Studies in primates are ongoing to assess its efficiency (Baur et al. 2006b). However more recent reports from other groups revealed their inability to reproduce these data showing again the discrepancies.

The use of animal models were very helpful in testing a myriad of nutritional supplements which were however not reproducible when performed in humans (Phelan et al. 2005). The most striking example is caloric restriction which is meant to extend lifespan et decrease the susceptibility to infections and cancers via the maintenance of a fully functional immune system. Caloric restriction can act via

ghrelin, a peptide produce in the stomach which stimulate appetite and control the production of growth hormones (Muccioli et al. 2004). Ghrelin also control the energy expenditure and can influence the immune system (Dixit et al. 2005). The expression of ghrelin and its receptor by T-cells suggest a functional role for this peptide on immune functions. Dixit et al. showed the ability of ghrelin to inhibit the production of pro-inflammatory cytokines such as IL-6 and TNF-α (Dixit et al. 2004). Thus, the modulation of ghrelin levels would influence the circulating cytokine levels. This has its importance when considering the role of inflame-aging in immune-senescence (Franceschi et al. 2000). Cytokines such as IL-6 play a critical role in the age-related low-grade inflammation. It is still unclear how to modulate ghrelin levels but this could be an interesting candidate in preventing immune-senescence and helping old individuals to recover appetite.

Our knowledge of other nutrients, such as lipids, has to increase to find any putative beneficial role for immunity. The quantitative and more importantly qualitative control of lipid consumption in aged individuals is rarely assessed. Due to the effect of lipids, especially those which are saturated, it would be of great interest to investigate the ways to improve immunity in immuno-compromised individuals. Some studies already demonstrated the ant-iinflammatory effect of omega-3 polyunsaturated fatty acids (Thies et al. 2001). The use of fish-oil validated the anti-inflammatory properties of such lipids, relevant to a variety of pathologies such as rheumatoid arthritis, Crohn's disease, psoriasis or systemic lupus erythematosus (Fritsche 2006; Harbige 2003). Whether fish-oil and other lipids decrease the low-grade inflammation seen with aging is unknown.

Recently, the beneficial effect of probiotics in milk-derived products on the immune system has been extensively advertised (Del Piano et al. 2004). Even if these agents showed in vitro-efficiency, it remains to be proved that it is also true in the in vivo situation. To reach this goal, the clinical trials will need to follow-up the participants for longer periods. The reasons for this are (i) to differentiate between short-term and long-term effects (ii) to determine the best frequency and duration of the supplementation (iii) to see any improvement in survival and disease-free periods (iv) to assess if it could only be a prophylactic or therapeutic supplementation (v) to circumvent any false-positive or false-negative results due to season-dependant dietary intake.

7 Conclusion

Preferably, the study of immune functions with aging and every intervention should be performed longitudinally (Pawelec et al. 2006). Aging is defined as over 65 years of age and nowadays people who retire may expect to live for a further 2 decades. Even if some nutritional compounds were proven to have an immunological beneficial effect, because of the increasing lifespan, this effect should be demonstrated as long-lasting. Short or mild-term effects are not sufficient anymore to be of interest for the young old population. It becomes more and more common to study popula-

tions over 75 years old (Wikby et al. 1994) while previous studies targeted the 65+ population only. The Swedish OCTO/NONA studies identified the Immune Risk Profile (IRP) of individuals over 80 years old. This IRP is defined by (i) an inversed CD4:CD8 ratio (ii) an increased frequency of CD8+CD28- T-cells (iii) a decreased number of B-cells and (iv) CMV seropositivity (Wikby et al. 2002). The next step will be to increase this study to other European countries and to longitudinally assess (Albers et al. 2005) the nutritional status of the IRP category to investigate any correlation between nutrition and the development of the risk profile.

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Role of Zinc and Selenium in Oxidative Stress and Immunosenescence: Implications for Healthy Ageing and Longevity

Eugenio Mocchegiani and Marco Malavolta

Contents

Abstract: Ageing is an inevitable biological process with gradual and spontaneous biochemical and physiological changes and increased susceptibility to diseases. Some nutritional factors (zinc and selenium) may remodel these changes leading to a possible escaping of diseases with subsequent healthy ageing, because they are especially involved in improving immune functions as well as antioxidant defense. Experiments performed "in vitro" (human lymphocytes exposed to endotoxins) and "in vivo" (old mice or young mice fed with low zinc dietary intake) show that zinc is important for immune response both innate and adoptive. Selenium provokes zinc release by Metallothioneins (MT), via reduction of glutathione peroxidase. This fact is crucial in ageing because high MT may be unable to release zinc with subsequent low intracellular free zinc ion availability for immune response. Taking into account the existence of zinc transporters (ZnT and ZIP family) for cellular zinc efflux and influx, respectively, the association between ZnT and MT is important in maintaining satisfactory intracellular zinc homeostasis in ageing. Improved immune

E. Mocchegiani (✉) · M. Malavolta
Immunology Ctr. section Nutrigenomic and Immunosenescence
Res Dept. INRCA
Via Birarelli 8
60121, Ancona, Italy
Tel.: +39-071-8004216; Fax: +39-071-206791
E-mail: e.mocchegiani@inrca.it

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performance occur in elderly after physiological zinc supplementation, which also induces prolonged survival in old, nude and neonatal thymectomized mice. The association "zinc plus selenium" improves humoral immunity in old subjects after influenza vaccination. Therefore, zinc and selenium are relevant for immunosenescence in order to achieve healthy ageing and longevity.

1 Introduction

Ageing is an inevitable biological process that is accompanied with gradual and spontaneous biochemical and physiological changes including increased susceptibility to diseases, adverse environmental conditions and loss of mobility and agility. Alterations in the immune functions play a fundamental role in ageing. The inability of an organism in remodeling these immune changes may lead to the appearance of some degenerative age-related diseases. As a result, the "remodeling theory of ageing" has been proposed (Paolisso et al. 2000). Various nutritional factors are directly linked with these phenomena as for instance in restoring the immune functions as well as in the capacity to respond to oxidative stress (Meydani 2001), which is in turn the main cause of the immune derangement in elderly (Pawelec 2000).

Approximately, 40 micronutrients (vitamins, essential minerals and other compounds required in small amount for normal metabolism) have been reported as essential components in the diet (Shenkin 2006). The dietary intake of essential macro and micronutrients is usually inadequate in the elderly (Ames 2006). Several causes contribute to this gap. First of all, the poor socio-economic condition present in a large part of old people may lead to a consumption of inexpensive foods deficient in micronutrients, such as carbohydrates (Kant 2000). The gap is worsened by loss of appetite, lack of teeth, intestinal malabsorption and decreased requirement of energy that lead to the final result of frailty, disability and mortality (Semba et al. 2006). Some authors have reported that the deficiency of macro and micronutrients in ageing is strictly related to global impairments of the immune functions with subsequent limited defense against external noxae and appearance of age-related diseases (Lesourd 2006). By contrast, recent longitudinal studies in dietary daily intake in human nonagenarian/centenarians (successful ageing) have shown that an adequate consumption of micro and macronutrients as well as a satisfactory content of some trace elements in the cells lead to good performances in several immune functions, especially in innate immune performances (Chernoff 2001; Mocchegiani et al. 2003). Therefore, nutritional factors may play a pivotal role for immunosenescence in order to reach healthy ageing and longevity. We herein review the role of zinc and selenium, taking into account the pivotal role played by these two micronutrients in the efficiency of the immune functions (Buttriss 2000). Recent epidemiological and clinical evidence have shown that in most developing countries deficiencies of these micronutrients are partly responsible for the severity of infectious disease, morbidity and mortality in malnourished children (Bhaskaram 2002) as well as in ageing (Meydani 2001). Indeed, these two trace elements form an important pillar in the nutrition

of young and elderly persons because also involved in tissue integrity (Enwonwu and Sanders 2001). Thus, it is evident that a deficiency of these elements could lead to a crucial impairment of the organ and tissue function with subsequent influence on many body homeostatic mechanisms, including the immune functions.

2 Zinc

2.1 Zinc Biology

Zinc is one of the most important trace elements in the body, although its presence in nature does not exceed 0.02% (Mills 1989). The major characteristics of zinc include a highly concentrated charge, a small radius (0.65A), no variable valence [low risk of free radical production], ready passage from one symmetry in its surroundings to another without exchange, rapid exchange of ligands (on and off reactions), and binding mostly to S- and N-donors in biological systems. These properties enable zinc to play a major biological role as a catalyst. Removal of the catalytic zinc results in an active apoenzyme that usually retains the native tertiary structure (Vallee and Falchuk 1993). Thus, it is not surprising that zinc is essential for the activity of more than 300 enzymes influencing the activity of zinc dependent antioxidant enzymes, such as superoxide dismutase (SOD) and various organ functions having a secondary effect on the immune system (Rink and Gabriel 2000).

Zinc also regulates the balance between the gene expression of metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs; Nagase and Woessner 1999). The main function of MMPs is the removal of extracellular matrix (ECM) during tissue resorption and progression of many diseases. However, it is notable that MMPs also alter biological function of ECM macromolecules by specific proteolysis (Shapiro 1998). Therefore, since MMPs are induced especially by proinflammatory cytokines (IL-1 and TNF-alpha), an overexpression of MMPs may lead to excessive proteolysis of ECM, as it occurs in chronic inflammation (Gueders et al. 2006). As a consequence, degradation of ECM and limited cell–cell adhesion may occur, so "trapping" bioactive mediators (Moot and Werb 2004). Thus, the expression of MMPs genes are under the control of some inhibitors of MMPs, such as TIMPs gene products, α-2 macroglobulin (α-2M) and 13-amyloid precursor protein (Nagase and Woessner 1999). As a result, a balance in the expression of the metalloproteinases [either as activators (MMPs) or as inhibitors (TIMPs)] is necessary for an optimal function of many biological systems. Examples of altering the balance between MMPs and TIMPs or α-2M have been recorded in certain types of cancer, infections and ageing (Nagase and Woessner 1999) that are conditions characterized by zinc deficiency (Fabris and Mocchegiani 1995). Zinc also regulates G0/G1 phase of cell cycle through Cyclins/CDK complexes in a dose dependent manner. Specifically, high doses of zinc (900 μM) result in cell cycle arrest (Paramanantham et al. 1996), whereas low doses of zinc (150 μM) inhibit apoptosis (Fraker 2005). Zinc is present in "zinc finger domains" of many proteins, peptides,

enzymes, hormones, transcriptional factors and cytokines, which act in maintaining body homeostasis (Coleman 1992; Berg and Shi 1996). Zinc also regulates mRNA stability (Taylor and Blackshear 1995) and extracellular matrix (Vallee and Falchuk 1993). Moreover, zinc binds enzymes, proteins and peptides with different binding affinity (kd) ranging from 10^{-2} to 10^{-14} mol/L (*See* review Mocchegiani et al. 1998). These compounds display low biological activity when the zinc-binding doesn't occur, as for instance for thymic hormone named thymulin, which loses its activity in absence of zinc (Fabris et al. 1984). Finally, zinc plays a critical role in structure, function, stabilization and fluidity of biomembrane due to its binding to sulphydryl groups forming mercaptides (Vallee and Falchuk 1993).

Zinc also maintains the enzymatic activity of inducible nitric-oxide synthase (iNOS; Bodgan et al. 2000), with a binding between zinc and two cysteine residues, which are part of the structures of the heme domain of iNOS (Li et al. 1999). As: (i) Nitric Oxide (NO), via NO synthases, affects the gene expression of metallothioneins (MT) in order to protect the host from oxidative stress (Arizono et al. 1995) and (ii) NO is involved in zinc release from MT, via s-nitrosylation (Zangger et al. 2001), the structural task of zinc in NO production is crucial.

In this context, the release of zinc by MT, via s-nytrosilation, contributing to raise the intracellular free zinc ions concentration, plays a crucial role in modulating the production of proinflammatory cytokines and in the activation of immune cells (Rink and Haase 2007). Therefore, the interrelationships between zinc and MT is crucial in maintaining the immune response especially in ageing where the production of proinflammatory cytokines is chronic leading to a constant presence of inflammatory status coupled with low intracellular zinc ion bioavailability (Mocchegiani et al. 2004). The interrelationship between zinc and MT is also regulated by the special proteins named zinc transporters (ZnT), which in turn appear to be also specifically involved through regulation of cellular zinc homeostasis via influx, efflux, or vesicular sequestration (Cousins and McMahon 2000; Eide 2006). The ZnT, some of which are tissue specific, maintain intracellular zinc concentration in a narrow physiological range in order to avoid cellular zinc toxicity or deficiency when dietary zinc intakes fluctuate. Two families of ZnT have been identified. The ZnT family decreases cytoplasmic zinc concentrations by secretion, sequestration, or efflux, whereas the ZIP family increases cytoplasmic zinc influx or release of stored zinc (Eide 2006). Therefore, the balance of ZnT is fundamental to maintain an optimal intracellular zinc homeostasis in ageing, because reduced zinc intake by the diet or intestinal zinc malabsorption or loss of zinc through urine by high levels of proinflammatory cytokines are usual events in elderly (Prasad et al. 1993b).

2.2 Zinc-Metallothioneins and Ageing

MT, are a group of low-molecular-weight metal-binding proteins who have high affinity for zinc (kd = 1.4×10^{-13} M; Kagi and Schaffer 1998). MT exist in different isoforms characterized by the length of aminoacid chain: isoform I, II, III e IV mapped on chro-

mosome 16 in man and on chromosome 8 in mice with complex polymorphisms (West et al. 1990). The more common isoforms are I and II; the isoform III, also called growth inhibitory factor (GIF), is a brain-specific member of the MT family and the isoform IV is restricted in squamous epithelia. MT contain 20 cysteines, all in reduced form, and bind seven zinc atoms through mercaptide bonds that have the spectroscopy characteristics of metal thiolate clusters (Maret and Vallee 1998). The zinc/cysteine clusters are of two different types. In the beta-domain cluster, three bridging and six terminal cysteine thiolates provide a coordination environment that is identical for each of the three zinc atoms. In the alpha-domain clusters, there are two different zinc sites; two of them have one terminal ligand and three bridging ligands respectively, while the other two have two terminal and two bridging ligands (Maret and Vallee 1998).

Following these biochemical characteristics, MT distribute intracellular zinc as zinc undergoes rapid inter- and intracluster exchange (Kagi and Schaffer 1998). Moreover, MT act as antioxidant since zinc-sulfur cluster is sensitive to changes of cellular redox state and oxidizing sites in MT (reduced thiol groups) induce the transfer of zinc from its MT binding sites to those of lower affinity in other proteins (Kagi and Schaffer 1998). This transfer confers biological activity to antioxidant metalloenzymes. Therefore, the redox properties of MT and their effect on zinc in the clusters are crucial for the protective role of MT in presence of ionizing and UV radiations (Cai et al. 1999), heavy metals (mercury, cadmium), lipid peroxidation, reactive oxygen species, oxidative stress caused by anticancer drugs, and conditions of hyperoxia (Sato and Kondoh 2002). This protective role of MT has been studied especially in young-adult MT knockout mice (null mice) for short periods of exposure to toxic metals, such as cadmium for 10 weeks (Habeebu et al. 2000) or mercury (one single injection and the effect of mercury analyzed 3 days after the injection; Satoh et al. 1997), or to anticancer agents for 48–72 hrs. (Kondo et al. 1997) or in presence of an excess of zinc or zinc deficiency for 3 weeks (Kelly et al. 1996). Therefore, the protective role of MT is evident in transient stress condition, as it may occur in young adult-age, in which the chronic status (by stress or inflammation) is a rare event (Mocchegiani et al. 2006). In contrast, this role may be questionable in ageing because the stress-like condition and inflammation by high levels of IL-6 are chronic (Ashok and Ali 1999), with also a different response to stress with respect to young (DeGroot et al. 2006). Since IL-6 affects the gene expression of MT (Hernandez et al.2000), these proteins may turn off from protective to harmful agents in ageing following the "antagonistic pleiotropy theory of ageing" (Williams and Day 2003). In fact, despite MT increase in ageing, a limited release of zinc by MT leading to an impaired immune and antioxidant response has been proposed (Mocchegiani et al. 2000a, b). In contrast, in presence of lower stress and inflammation, as it occurs in centenarians, MT production is low coupled with satisfactory zinc ion bioavailability (Mocchegiani et al. 2002a). Indeed, since IL-6 acts on the cells through its subunit receptor gp130 (Bravo and Heath 2000), the relative lower gene expression of gp130 with respect to elderly found in centenarians (Moroni et al. 2005) may imply that a quota of IL-6 is inactive in centenarians leading to low gene expression of MT, satisfactory free zinc ion availability and low degree of inflammation (Mocchegiani et al. 2002a). As a result, the satisfactory immune performances and antioxidant activi-

ties lead to a good healthy status in these exceptional individuals (Mecocci et al. 2000; Mocchegiani et al. 2002a). Therefore, the interrelationships among inflammatory status, MT and zinc are pivotal in order to achieve successful ageing, furtherly suggesting a different role of MT in ageing that is crucial for immune response (Mocchegiani et al. 2000a). Whether MT might play an antagonistic pleiotropic role remains however to be clearly demonstrated also taking into account that they may play different role in different organs. On this aspect, recent findings in cardiac-specific Metallothionein transgenic mice suggest that the expression of these proteins in cardiocytes may alleviate aging-induced cardiac contractile defects and oxidative stress prolonging life span (Yang et al. 2006). In addition, Daf-2 mutant nematodes other than a longevity phenotype, display an altered expression of MT which, in turn, seems to interact with the insulin signaling pathway (Barsyte et al. 2001). Therefore, even if the specific function of MT in ageing is still a matter of discussion, all these reports associated to recent findings on the possible role played by MT in modulating cellular respiration and energy metabolism (Feng et al. 2005; Ye et al. 2001) strongly suggest that these proteins are involved in the maintenance of health status and in successful aging. On the other hand, recent findings show a novel polymorphisms of MT1A (A/C at position +647 leading to an asparagine/threonine aminoacid substitution) involved in successful ageing, lower inflammation and satisfactory intracellular zinc ion bioavailability (Cipriano et al. 2006).

2.3 Zinc Transporter and Ageing

With regard to the role played by the ZnT in ageing and immunosenescence, a paucity of data exists in literature. After an increase from the birth up to adult age in some tissues, pancreas (Clifford and MacDonald 2000) or brain (Nitzan et al. 2002), significant decrements of both ZnT and ZIP families in peripheral leukocytes from elderly women occur, in particular the subtypes ZnT1 and ZIP1 (Andree et al. 2004). Taking into account that ZIP family increases cytoplasmic zinc influx (Eide 2006), an intriguing point is that Zip14 expression is up-regulated through IL-6, and that this zinc transporter most likely plays a major role in the mechanism responsible for an excess of intracellular zinc and, at the same time, for hypozincemia that accompanies the acute-phase response to inflammation and infection (Liuzzi et al. 2005). Since chronic inflammation by high IL-6, hypozincemia and risk of infections are usual events in old age (Mocchegiani et al. 2003), the possible alterations of the ZnT in ageing coupled with the inability of high zinc-bound MT in zinc release, may thus allow still more synergistic deleterious effects on immune response that it may be due or to low or excess of zinc within the cells. This last assumption is supported by the discovery that both low and high levels of intracellular zinc lead to cell death (Fraker 2005). Therefore, the intracellular zinc ion availability should be maintained within a strict range in order to exert beneficial effect, otherwise it may trigger pathological pathway cascades possibly contributing to the onset and progression of degenerative diseases (Mocchegiani et al. 2006).

2.4 Zinc-MT and Immunosenescence

For a prompt immune response against stressor agents and inflammation, macrophages produce some cytokines, such as IL-1, IL-6, IFN-α, TNF-α, which, in turn, provoke a new synthesis of MT in the liver but, at the same time, an alteration in the zinc status (Bui et al. 1994). These findings clearly suggest the existence of interplay between MT and the immune system. IL-1 affects MT mRNA in thymic epithelial cells (TECs) by means of PKC, which is, in turn, zinc-dependent (Coto et al. 1992) and participates in metal-induced MTmRNA (Yu et al. 1997). Moreover, MT are donors of zinc for thymulin reactivation in TECs (Coto et al. 1992). MT act both as a reservoir of zinc during zinc deficiency and as a zinc buffering protein in presence of excessive amount of zinc in order to prevent zinc toxicity (Kelly et al. 1996). Following these findings, MT are, out of doubt, protective agents with also the task in preventing zinc deficiency during an inflammatory status. It has been recently reported that, under inflammatory conditions, MT in the extracellular environment may support the beneficial movement of leukocytes to the site of inflammation representing a "danger signal" for the immune cells and modifying the character of the immune response when cells sense cellular stress. However, high MT produced in chronic inflammation, may alter the normal chemotactic responses that regulate leukocyte trafficking (Yin et al. 2005). Taking into account that zinc ions attract leukocytes by inducing and promoting the chemotactic response (Hujanen et al. 1995), high MT production might be dangerous for immune response in presence of chronic inflammation. Moreover, (i) the existence of high MT and low zinc ion bioavailability in the atrophic thymus from old mice (Mocchegiani et al. 2004); (ii) the presence of high MT in lymphocytes from old people and Down's syndrome subjects (syndrome of accelerated ageing) coupled with impaired innate immunity (Mocchegiani et al. 2002a) and (iii) the occurrence of atrophic thymus in young stressed mice overexpressing MT (Mocchegiani et al. 2002b), furtherly suggest this dangerous role played by MT in immune function during ageing. Additionally, elevated levels of extracellular MT, as it can be found especially in chronic inflammatory sites, can cause a dramatic decreases in cytotoxic T lymphocyte (CTL) activity against allogeneic target cells, reduces the proliferative response of CTLL-2 cells to cytokines, and decreases the level of major histocompatibility complex (MHC) Class I and CD8 molecules detectable on the surface of lymphocytes (Youn and Lynes 1999). Therefore, high MT may also have an immunosuppressive effect worsened by the fact they are not donors of zinc in ageing but rather sequester zinc. On the other hand, high MT induce down-regulation of many other biological functions related to zinc, such as metabolism, gene expression and signal transduction (Kagi and Schaffer 1998). An unbalance between MT isoforms leads to impairments of zinc-dependent body homeostatic mechanisms within the brain, as reported in SAMP10 mice (model of accelerated ageing; Wen et al. 2006). Moreover, high MT are an index of unfavorable prognosis in cancer (Ebadi and Swanson 1988).

However, the limited capability of MT in zinc release is still unresolved problem in ageing, especially regarding to the precise mechanism involved. The zinc release

from MT under oxidative stress conditions is accompanied by more MT disulfide bond formation (Feng et al. 2006). But, an intriguing point is that also NO provokes the zinc release by MT, via s-nitrosylation (Zangger et al. 2001). Despite iNOS increases in ageing, the release of zinc by MT is very limited. One hypothesis might be an unbalance between NO synthases (iNOS and cNOS; Mocchegiani et al. 2000a). However, NO donors and zinc fluorescent probes are useful tools in order to study the zinc release from MT and to evaluate the intracellular labile zinc in ageing.

Using a methodology for testing intracellular free zinc ion availability in PBMC recently developed in our laboratory (Malavolta et al. 2006), it has been shown that the NO-induced release of zinc can be preserved at least in nonagenarians carrying MT1A polymorphism favorable for successful ageing (Cipriano et al. 2006). Moreover, a flow cytometric assay for the measurement of intracellular labile zinc was recently developed by Haase et al. (2006) The zinc-sensitive fluorescent probe named FluoZin-3 was used to quantify the amount of labile zinc in peripheral blood mononuclear cells isolated from human blood. With this method, the intracellular concentrations of labile zinc in resting cells were estimated to be 0.17 nM in monocytes and 0.35 nM in lymphocytes (CD4+; Haase et al. 2006). Therefore, the combination of these two novel methodological procedures will permit to study in depth the cause of limited zinc release from MT in ageing and, at the same time, to evaluate the intracellular labile zinc. Anyway, a limited zinc release from MT exists in ageing provoking a low free zinc ion availability for immune response and antioxidant activity. The recent discovery of another novel polymorphism of MT (-209A/G MT2A) may indirectly support this assumption. Indeed, old subjects carrying AA genotype display high MT, low zinc ion availability, enhanced IL-6 and impaired innate immune response with subsequent possible risk for atherosclerosis and diabetes type II (Giacconi et al. 2005). Therefore, MT may have a different role in immunosenescence, following the concept that several genes/proteins that increase fitness early in life may also have negative effects later in life: named “Antagonistic Pleiotropy Theory of Ageing”(Williams and Day 2003).

2.5 Rationale for Zinc Supplementation in Ageing: “In Vitro” Studies

Since the crude zinc balance is negative in old mice (Mocchegiani et al. 1995) and in old human (Turnlund et al. 1986), zinc supplementations in old mice and in elderly have been carried out in order to improve the immune response. The scientific rationale for the immune supporting role of zinc supplementation “in vivo” finds consistent support by data obtained “in vitro” in immune cells.

At this regard, many effects of zinc on immune cells have been shown by assessing the cytokine concentration in the samples after zinc stimulation. When PBMCs are stimulated with zinc, IL-1, IL-6, TNF-α, soluble (s)IL-2 receptor and IFN-γ are released (Ibs and Rink 2003). The secretion of IL-1, IL-6 and TNF-α is induced directly by zinc in monocytes and is independent by the presence of

lymphocytes (Driessen et al. 1994). However, the effect of zinc on monocytes may depend upon external stimulation. In fact, zinc inhibits LPS-induced TNF-α and IL-1β release from primary human monocytes and monocytic cell lines through the inhibition of cyclic nucleotide phosphodiesterase activity (von Bulow et al. 2005), suggesting that zinc may display also some anti-inflammatory properties.

The dose of zinc used is also a critical variable. In serum-free culture medium, concentrations >100 μM of zinc/L stimulate monocytes but prevent T-cells from activating, perhaps due to the lower intracellular content in T-cells than in monocytes (Ibs and Rink 2003).

Treatment with zinc "in vitro" generally displays also beneficial effects on cell survival but, the effect largely depends upon the cell type and the dose of zinc used. It seems that both apoptosis prevention and induction are mediated by pathways involving zinc and/or zinc-dependent enzymes (Clegg et al. 2005; Wiseman et al. 2006). Therefore, the modulation of the zinc homeostasis plays a key role not only in preventing apoptosis, when oxidative stress is low, but also in inducing apoptosis, when oxidative stress and cellular damage is high, in order to down regulate immune responses and to eliminate virally infected or malignant cells (Fraker and Lill-Elghanian 2004). Taking into account the strict correlation existing between oxidative stress and immune function especially in response to specific stimuli through the production of proinflammatory cytokines for a prompt immune response (Franceschi et al. 2005), this role of zinc in inducing apoptosis of only damaged cells in presence of high oxidative stress is evident in young-adult age and with a great surprising in very old age (Ostan et al. 2006), perhaps due to the presence of satisfactory zinc ion availability (Mocchegiani et al. 2002a) that regulates p53 activity for health lifespan (Bauer and Helfand 2006), being p53 a zinc binding protein (Hainaut and Mann 2001).

Experiments in thymocytes also support this point of view, since media supplemented with zinc from 50 up to 150 μM prevents old thymocyte apoptosis induced by dexamethasone or serum deprivation (Provinciali etal. 1998), whereas the direct introduction of free zinc as zinc-pyrithone inside thymocytes induces apoptosis (Mann and Fraker 2005). In this last case, the continuous presence of intracellular free zinc ions can advice the cell that permanent oxidative stress and irreversible damage are present, thus activating proapoptotic pathways.

2.6 Effect of Zinc Supplementation in Ageing

2.6.1 Old Mice

Old literature reports that a physiological zinc supplementation in the diet throughout the life span in adult rodents prevents some age-related cell-mediated immune modifications, such as the decreased circulating thymic hormone levels (Iwata et al. 1979). More recently, a physiological zinc supplementation (18 μg/ml Zn^{++} in the

drinking water for 1-month) in old mice induces thymus re-growth and functionality (Dardenne et al. 1993; Mocchegiani et al. 1995) and restoration of NK cell cytotoxicity (Mocchegiani et al. 1995). That the benefit of zinc supplementation upon the immune functions in old mice is not to consider an epiphenomenon comes by the analysis of the rate of survival in old zinc treated mice. Old mice (inbreed Balb/c mice) treated with daily zinc at the dose reported above in drinking water from the pre-senescent age (12–14 months of age) display a significant increment of the rate of survival up to 33th month of age when this strain of mice usually lives up to 28–29th month of age. The increment of old survivor zinc treated mice is particularly significant in the middle age (24–25th month of age; Mocchegiani et al. 2000b). The increased rate of survival is largely due to significant decrements of deaths due to cancer and infection in the middle age (Mocchegiani et al. 2000b). Of interest, the crude zinc balance is negative, other than in old mice, also in nude and neonatal thymectomized mice (Mocchegiani et al. 1995 2000b 2007). A zinc supplementation increases the rate of survival also in nude and neonatal thymectomized mice (Mocchegiani et al. 2007), which display a very short survival due to thymus absence (Piantanelli and Fabris 1978). Taking into account that the liver extrathymic T-cell pathway is prominent in nude, thymectomized and old mice in order to compensate the thymic failure (Abo 2000), it is evident the zinc also affects the liver extrathymic T-cell pathway with good performances of the immune functions against external noxae (Mocchegiani et al. 1998) coupled with increased rate of survival.

2.6.2 Elderly

With regard to elderly, undefined data exist on the beneficial effect of zinc supplementation upon the immune efficiency due to different doses of zinc used and to the length of the treatment (Bodgen et al. 1990; Boukaiba et al. 1993; Cakman et al. 1997; Duchateau et al. 1981; Fortes et al. 1998; Prasad et al. 1993b; Sandstead et al. 1982). Although zinc was used at the dose recommended by RDA (from 15 to 25 mg/day) in the majority of the studies, Prasad et al. (1993b) and Boukaniba et al. (1993) have found an increment of thymulin activity and improvements in response to skin-test antigens and taste acuity (zinc dose = 15 mg /day for 4 months); Bodgen et al. (1990) have reported no benefit exclusively for increased lymphocyte mitogen proliferative response (zinc dose = 15 mg/day for 1-year); Cakman et al. (1997) have found enhanced IFN-γ production by leukocytes (zinc dose = 15 mg/day for 45 days); Fortes et al. (1998) report an increased number of CTLs (zinc dose = 25 mg/day for 40 days); Duchateau et al. (1981) and Sandstaed et al. (1982) have observed an improvement in response to skin-test antigens and taste acuity (zinc dose = 220 mg/day for 1-month). Thus, it seems evident from these studies that physiological dose of zinc for a long period or high doses of zinc for short periods might induce limited effects on immune response perhaps due to a zinc accumulation in various organs and tissues with subsequent toxic effect of zinc upon the immune functions (Fosmire 1990; Sandstead 1995). In this context, it is useful to remind that high

doses of zinc trigger apoptosis of the immune cells in presence of high oxidative stress, as reported above. Therefore, zinc supplementation has to be used with caution for short periods and on alternate cycles. Following that, in our experience, zinc treatment at the dose of 15 mg Zn^{++}/day for 1-month in Down's syndrome subjects, in elderly and in old infected patients restores thymic endocrine activity, lymphocyte mitogen proliferative response, CD4+ cell number, peripheral immune efficiency (NK cell cytotoxicity), Th1/Th2 paradigm (Franceschi et al. 1988; Kahmann et al. 2006; Mocchegiani et al. 2003) and DNA-repair (Chiricolo et al.1993). At clinical level, significant reductions of infection relapses occur in Down's syndrome (Licastro et al. 1994) in elderly and in old infected patients with a faster outcome from the pathology (Mocchegiani et al. 2003).

Physiological zinc supplementation was reported to lead to a decrement in plasma lipid peroxide concentrations in elderly people living in a public home (Fortes et al. 1997). The positive effect of zinc on lipid peroxide could derive from its protective effects on sulphydryl groups against oxidation and the fact that zinc is a component of superoxide dismutase (SOD; Mills 1989).

Zinc supplementation is also useful in reducing the oxidative stress in old patients with diabetes type II (Roussel et al. 2003) because it inhibits NF-kB activation and decreases inducible NO synthase. As such, the generation of ROS decreases, thus zinc provides a protective effect on β cells against death (Ho et al. 2001).

An intriguing point of the zinc supplementation is the increment of ZnT. Elderly women treated for 27 days with 22mg of zinc gluconate /day display significant increments of ZnT1 gene expression in peripheral leukocytes (Andree, et al. 2004), even if the gene expression of the ZnT is sensitive in relation to the immune cells considered (Whitney et al. 2003). Such increments of ZnT1 have been also observed in human lymphoblastoid cells adding in vitro 50 or 100 μmol/L of zinc (Andree et al. 2004), furtherly suggesting the relevance of zinc supplementation also in affecting the gene expression of ZnT and, consequently, the correct maintenance of intracellular zinc homeostasis.

That the beneficial effects of zinc supplementation are not to be considered as epiphenomena, it comes by the increased survival also in nude and neonatal thymectomized (nTx) mice treated with physiological zinc (18 μg Zn++/day for 1-month) in the drinking water, taking into account that they display a very short survival due to thymic absence and negative crude zinc balance (Mocchegiani et al. 1995, 2002b, 2007). The prolonged survival is largely due to mortality reduction (about 50%) by infections because zinc also affects the extrathymic T-cell pathway that is prominent in old, nude and nTx mice for T-cell maturation and host defense (Abo et al. 2000). Indeed, in vivo and in vitro studies have shown that zinc is a key trace element for liver T-cell maturation and function, particularly for liver NKT cells bearing TCR γδ with high production of IFN-γ (Mocchegiani et al. 2004). Of interest, the increment and function of NKT cells (Miyaji et al. 2000) and Tγδ cells (Colonna-Romano et al. 2002) also occur in human centenarians, who in turn display satisfactory zinc ion bioavailability and good immune response (Mocchegiani et al. 2002a).

All these "in vitro" and "in vivo" studies in ageing, some age-related diseases, and syndrome of accelerated ageing (nude mice, nTx mice, Down's Syndrome)

demonstrate the pivotal role played by zinc supplementation in maintaining or improving global immune response and in fighting the oxidative stress, strengthen by findings observed in human centenarians.

However, since zinc also affects MT gene expression (Maret 2003), the question arises whether zinc supplementation in old age may furtherly increase MT causing possible major harmful effects. Old zinc treated mice exhibit no further significant increments of liver MT mRNA, suggesting that MT in ageing may be already over-expressed before supplementation (Mocchegiani et al. 2002b). Moreover, the effects observed during zinc supplementation on the immune system, such as reduced inflammation and restored Th1/Th2 paradigm (Prasad 2000), suggest that intracellular zinc may return available despite over-expressed MT (Mocchegiani et al. 2002b) with a maintenance of their original protective role. Therefore, the possible harmful effect of MT in ageing seems to not constitute a problem during physiological zinc supplementation.

2.7 Zinc Interaction with Other Micronutrients and Zinc Toxicity

The beneficial effect of physiological zinc supplementation must be, however, related to the levels of other cations such as cadmium, lead, calcium, iron, manganese and copper. The beneficial effects of zinc on ameliorating toxicity of cadmium and lead, accentuation of zinc deficiency by administration of calcium and phytate, and production of hypocupremia by excessive zinc intake in humans and animals, are some examples of competition phenomena between these cations (Hill 1976). Such a competition occurs because these ions have similar valence shell electronic structure and, therefore could be antagonist to each other. For instance, the competition between zinc and iron (Fe++) occurs at the level of cysteine-histidine ligands for the formation of iron or zinc "fingers" proteins (Prasad 1993a). If iron is excess, a preferential binding of iron than zinc to the metal free-protein occurs. Excess of zinc or zinc deficiency impairs DNA-protein interactions of zinc-fingers domains with their cognate DNA target sites. In these conditions the production of some transcriptional factors like SP1 or TFIIIA is impaired (Thiesen and Bach 1991). The same impairment of zinc fingers DNA domains occurs in excess or deficiency of copper (Prasad 1993a). This reinforces the notion of the relevance of interactions between zinc and copper as well as with other metals in the immune efficiency (Sandstead 1995). Thus a limited range of bioavailability exists for each metal. As such, immune responses are optimum. Indeed, the beneficial effect of zinc is strictly dependent by the dose and the length of treatment. Zinc accumulation or imbalance zinc-to-copper ratio may occur despite low doses of zinc (Fosmire 1990). As such, harmful side effects in the cardiovascular system and in the brain may appear with increased low-density lipoprotein and cholesterol (Fosmire 1990) and neural cell-death (Kim et al. 1999), respectively. Therefore, caution in zinc supplementation is necessary for avoiding undesirable and harmful unexpected side effects. Zinc supplementation must not exceed 2–3 times the RDA/day, for short periods

(1–2 months) and on alternate cycles. This treatment doesn't interfere in copper absorption (Faillet-Coudray et al. 2006; Licastro et al. 1994). Zinc picolinate form may be the best supplement (Wapnir et al. 1983).

3 Selenium

3.1 Selenium Biology

Selenium (Se) is an essential dietary element for the prevention of some diseases, including cancer and infections (Schwarz 1976). Such an assumption has been subsequently confirmed in animals with a selenium deficiency in the diet and concomitant treatment with various carcinogens, such as 1,2-dimethylhydrazine (DMH) or dimethylbenz(a)anthracene (DMBA), compared with animals fed with higher content of selenium in the diet. In this context, although Se deficiency appears to affect DMH toxicity with however no inhibition of tumor development by nutritional Se (0.1 ppm Se; Pence and Buddingh 1985), three relevant papers report a greater development of carcinoma by DHM or DMBA in various organs (colon and mammary gland) in rats fed with selenium deficiency in the diet in comparison with rats treated with 5 ppm of Se (Jacobs 1983; Liu and Milner 1992; McGarrity and Peiffer 1993). These findings further suggest the ability of dietary selenium to inhibit the in vivo metabolism of carcinogens DMBA or DMH with subsequent less development of the tumor. With regard to infection, decreased dietary selenium can change a normally avirulent B3 coxsackievirus (CBV3/0) into a virulent virus (CBV3/20) by inducing changes in viral genoma, especially in viral RNA polymerase mutations (Duarte et al. 1994) that infect heart muscle and cause myocarditis with subsequent possible development of dilated cardiomyopathy and death (Beck and Levander 2000). In food, selenium derives from vegetables and animal products and in particular from the consumption of seafood, liver, and cereals. However, in vegetables and cereals the amount of selenium varies in soil in different countries and geographical regions (Wasowicz et al. 2003). Indeed, selenium deficiency and related diseases have been well documented in geographic regions where the soil content is low, such as the Chinese province of Keshan (Li et al. 1985). From this Region of China, in fact, Keshan disease is named the pathology characterized by selenium deficiency and presence of substantial number of virulent viruses, including coxsackieviruses (Li et al. 1995).

Mammals can use both inorganic and organic selenium as a nutrient. Most of the biological functions of selenium are attributed to selenoproteins, which contain selenocysteine residues responsible for their specific activity. Selenoproteins are present in every cell type. The human selenoproteome consists of 25 selenoproteins, mostly involved in antioxidant defence systems (Kryukov et al. 2003).

Glutathione peroxidases (GPxs), a family of the selenoproteins, protect cells against oxidative damage by catalysing the reduction of hydrogen peroxide and other hydroperoxides (Brigelius-Flohe 1999; Hall et al. 1998). Five selenium dependent

GPx isoforms exist in humans and four isoforms in mice. GPx1 is found in the cytosol of almost all cells and catalyses the reduction of free hydroperoxides. GPx2 is expressed in the gastro-intestinal tract and has a substrate specificity similar to GPx1; GPx3 is an extracellular enzyme found in plasma and reduces membrane-bound phospholipid hydroperoxides (Brigelius-Flohe 1999). GPx4 is expressed in various tissues, and reduces phospholipid hydroperoxide and hydrogen peroxide using also thiols, such as 2-mercaptoethanol, cysteine and homocysteine, other than GSH as reductant agents (Roveri et al. 1994). The isoform GPx6 seems to be specifically expressed in embryonic tissues and olfactory epithelium (Kryukov et al. 2003). It also exist a selenium independent isoform, GPx5, which is an epididymis isoenzyme present in mice and humans (Hall et al. 1998), but its mRNA was found to be not translated into functional protein in human epididymis (Ghyselinck et al. 1993). Selenium is also involved in the thioredoxin system, a major enzymatic system that plays an important role in maintaining the redox state of the cell (Holmgren 1985). This system is highly complementary to the GSH system in protecting against oxidative stress (Watson et al. 2004). It comprises basically of thioredoxin (Trx) and the selenoprotein thioredoxin reductase (TR) and uses the reducing power of NADPH to act as a potent antioxidant system as well as a general disulfide redox system (Rundolf et al. 2004). Mammalian TR maintains Trx in a reduced state (Holmgren 1985) and reduces a variety of other substrates including nondisulphides. The thioredoxin system protects the cell against oxidative stress through a variety of mechanisms. Trx can directly quench singlet oxygen and scavange hydroxyl radicals (Das and Das 2000), or reduced Trx can indirectly serves as an electron donor for Trx peroxidase. In addition, human TR is directly capable to efficiently reduce lipid hydroperoxides, hydrogen peroxide and organic hydroperoxides using NADPH, especially in the presence of catalytic amount of selenocysteine, thus serving as an important alternative to the Gpx pathway for the elimination of harmful hydroperoxides (Bjornstedt et al. 1995). Trx system is also critical for signal transduction (Arner and Holmgren 2000) and in the restoration of the reduced form of several antioxidant compounds, including ascorbic acid, lipoic acid, and ubiquinone (Nordberg and Arner 2001). In this context, selenomethionine, a potent catalytic antioxidant in biological system and an aminoacid occurring in proteins in place of methionine (Walter and Roy 1971), reacts more efficiently than methionine (Padmaja et al. 1996) with oxidants forming methionine selenoxide which, in turn, is effectively and rapidly reduced to seleniomethionine by glutathione (Assmann et al. 1998). In contrast, methionine sulphoxide that it is produced by the oxidation of methionine in presence of oxidants, is not simply reduced by GSH, but it requires a specific enzymatic reaction catalyzed by methionine sulphoxide reductase (Levine et al. 1996). Since selenomethionine can occur in proteins such as haemoglobin (Beilstein and Whanger 1986), these residues may play a defensive role against peroxinitite.

Another selenoprotein, which reduces phospholipid hydroperoxides in the presence of thiols, is the Selenoprotein P (SeP; Burk et al. 2003). SeP is expressed in many tissues and represents the major plasma selenoprotein, which contains 50% of the total plasma selenium in the form of selenocysteine. SeP protects endothelial

cells against damage from peroxynitrite and transports selenium from the liver to peripheral tissues (Burk et al. 2003).

Last, but not the least in order of importance, is a class of selenoproteins (iodothyronine deiodinase enzymes), which catalyse the peripheral deiodination of thyroxin (T4) to 3,3'5-triiodothyronine (T3). These enzymes play crucial roles in determining the circulating and intracellular levels of T3 and, consequently, the control of growth, development, differentiation, metabolism and finally also the immune response (Kohrle 2000; Beckett and Arthur 2005).

Immunologically, the ability of selenoproteins to protect the host from oxidative stress is vitally important, since many host defence systems rely on the microbiocidal effects of macrophage- or neutrophil-generated free-radical species. Oxidative species are generated through general metabolism, during the metabolism of xenobiotics and during exposure to ultraviolet radiation (UV) in sunlight. Inflammation as a process to clear infection and damaged tissue also generates great oxidative stress. If antioxidant systems are not functioning correctly, host cells will be damaged (McKenzie et al. 1998). Taking into account that the inflammation is chronic in ageing as well as oxidative stress (Franceschi et al. 2000), the role played by selenium through the selenopreoteins in immune response is therefore vital in elderly.

3.2 Selenium and Immune Function

The influence of selenium on the immune function can be, in part, attributed to the same selenoproteins involved in the protection against oxidative damage and, in part, to still undefined biochemical pathways. The antioxidant GPxs have probably a role in protecting neutrophils from ROS that are produced during inflammation (Arthur et al. 2003a, b). Selenium supplementation, in mice, increases the expression of subunits alpha (p55) and/or beta (p70/75) of IL-2 receptor (IL-2R) from activated lymphocytes and NK cells, thereby enhancing proliferation and clone expansion of cytotoxic precursor cells. In vitro, selenium enhances the release of tumor necrosis factor (TNF), IL-1 and IL-6 from LPS stimulated macrophages (*See* review Beckett et al. 2003). However one of the most widely investigated associations between selenium and the immune system is the effect of the micronutrient on neutrophil function. Neutrophils produce superoxide-derived radicals to take part in killing of microbes. This type of process is a balance between the production of sufficient radicals to kill invading organisms and the systems that protect the neutrophils themselves from the radicals. Thus, although selenium deficiency does not affect neutrophil numbers in a range of species, certain aspects of their function are defective (Turner and Finch 1991). Neutrophils from selenium-deficient mice, rats and cattle are able to ingest pathogens in vitro but are less able to kill them than are neutrophils from selenium-sufficient animals. This defective function has been associated with decreased cytosolic GPx (GPx1) activity in the neutrophils, which allows the free radicals that are produced in the respiratory burst to kill the neutrophils themselves (Arthur et al. 2003b).

Therefore, taking into account all these mechanisms, selenium deficiency has been mainly studied in relation to ageing/mortality and in some age-related diseases, whose pathogenesis is related to preservation of membrane integrity and to oxidative damage of biomolecules, such as lipids, lipoproteins and DNA.

3.3 Selenium, Ageing and Age-related Diseases

Selenium deficiency is a condition, mainly attributed to low selenium content in the soil or to long-term parenteral nutrition. Selenium is essential for several biochemical mechanisms and selenium blood decline concentrations relate to chronic age-related disease such as cancer, cardiovascular disease and immune dysfunctions (Seiler 2001). During ageing, selenium deficiency may occur in relation to intestinal malabsorption. However, few data report a marked selenium deficiency in old subjects (Seiler 2001). More recently, a paper has explored the relationships between plasma selenium and mortality in an elderly population for a long period of observation (9 years): the EVA (Etude du Vieillissement Artériel) study (Akbaraly et al. 2005). The authors have observed during this long period that the mortality rates were significantly higher in individuals with low selenium [1.01 μmol/L: a value below the cutoff considered as optimal (1.25–1.50 μmol/L; Thomson 2004; Combs 2001)]. When the underlying causes of death were considered, an association with low selenium and cancer-related mortality was found. The same authors suggest that plasma selenium could be an indicator of longevity in a preaging, independently living population not specifically at risk for cancer and cardiovascular diseases. Survival curves illustrate that the relationship between plasma selenium and mortality remained pertinent during the entire 9-year period (Akbaraly et al. 2005). However, the mechanism of this potential relationship is still under debate and further research needed especially on the role played by selenoproteins on this phenomenon. Other authors demonstrate selenium deficiency in elderly people in relation to hypothyroidism (Oliveri et al. 1996). Interestingly, human healthy centenarians display selenium values quite similar to normal elderly (Savarino et al. 2001). As few trials have been carried out up to date in elderly, it is difficult to report a specific beneficial effect of selenium in immunosenescence, even if beneficial effects of selenium supplementation on lymphocyte mitogen responsiveness have been reported in institutionalized elderly individuals (Peretz et al. 1991) and in old animals (Roy et al. 1995). Moreover, a Finnish study adding selenium to fertilizer has shown only an increased selenium status in the general population (young, adult, old; Aro et al. 1995), but not on its possible beneficial effects. The major evidence of the beneficial effects of selenium relate to age-associated diseases. Many studies have investigated the effects of selenium in carcinogen-exposed animals showing a reduction in tumor incidence and/or preneoplastic endpoints (Reid et al. 2002). A supplementation with 200 μg/day of organic selenium in randomized subjects showed preventive effects in the incidence and the mortality from various types of cancer (prostate, colorectal and lung cancer; Clark et al. 1998; Reid et al. 2002). Another large supplementation

trial in which a physiological amount of selenium (50 μg) was recently performed in Lixian (North China) in order to test its possible beneficial effect in preventing cancer. A small but significant reduction in total and cancer mortality was observed in subjects receiving selenium supplement. The reduction were shown to be greater in women than men and interestingly more pronounced in persons under the age of 55 years compared to individuals older than 55 years (Blot et al. 1995). Considering these results, it can be assumed that younger persons might be more amenable to a protective effect of selenium supplementation with thus a role of selenium in preventing age-related diseases or in enhancing the innate immune defenses in the course of the pathology, as observed in selenium supplemented cancer patients (200 mg/d of sodium selenite; Kiremidjian-Schumacher et al. 2000).

The relevance of selenium in the etiology of cardiovascular diseases has been also studied. Selenium metabolism is potentially involved in several protective biochemical pathways related to cardiovascular disease, such as reduction of LDL levels and lipoprotein oxidation, inhibition of foam-cell formation and shift in prostaglandin production from prostacyclin to tromboxane (Alissa et al. 2003). However, Wei et al. (2004) found no association between death for cardiovascular diseases and baseline selenium status in a cohort with a mean serum concentration of 0.93 μmol/L in younger individuals (mean age, 57 years). The major studies on the incidence of cardiovascular diseases in these last 5 years have been performed in adult people using a combinations of multivitamins and some trace elements, including selenium, as possible prevention of cardiovascular diseases (atherosclerosis, myocardial infarction, thrombosis). All these studies have shown a less incidence of cardiovascular diseases after supplementation with these combinations in comparison to placebo groups (Czernichow et al. 2005; Shenoy et al. 2006). Therefore, the existence of a clear link between selenium deficiency "in se" and cardiovascular disease remains to be clearly defined.

Finally, an intriguing point is the association between selenium deficiency, immune response and increased incidence of infections in adults and elderly. Patients with systemic inflammatory response syndrome display a strong impairment in immune efficiency, a decrease of 40% in plasma selenium concentrations coupled with increased morbidity and mortality rates (Forceville et al. 1998). The interrelationships between selenium deficiency, impaired immune response and infections have been clearly shown in experimental animals. An inoculated avirulent virus in selenium deficient animals turns into a virulent one due to genomic changes within the virus, provoking an impaired humoral immune defence (Beck 1999). In humans, a relevant clinical trial with multivitamins and selenium has shown an increment of CD4+ counts over the baseline levels (Coodley 1995) and enhanced GPx and GSH activity (Delmas-Beauvieux et al. 2006) in HIV infected patients This finding suggests that cysteine/GSH are effective natural inhibitors/combaters of (AIDS) viruses and thereby capable in preventing the development of chronic virus diseases that can lead to AIDS (Rayman 2000). Moreover, supplementation with multivitamins and trace elements, including Se, during treatment of pulmonary Tuberculosis may reduce mortality in subjects co-infected with HIV (Range et al. 2006). Therefore, an enhanced oxidative stress, caused by selenium deficiency, is the reason of

possible viral genetic changes (Beck et al. 2003) and increased progression of viral infections with subsequent impaired immune defense (Daniels 2004).

Additionally, it is also of interest for the role played by selenium deficiency in viral infections the following points: (i) the emergence during these last 4 years (from 2003) of a newly recognized human disease agent (coronavirus) that causes SARS from Guangdong Province of China (Lashley 2006) as well as from Northern Vietnam (Reynolds et al. 2006), where significant areas of overt selenium deficiency exist (Xia et al. 2005); ii) the increased risk of enhanced virulence of influenza virus in elderly (Ellis et al. 2003) associated with a possible selenium deficiency (Seiler 2001). Therefore, the selenium deficiency may be considered as a relevant risk factor for the appearance of age-related diseases (cancer, cardiovascular diseases and infections by viruses, which may become more virulent or mutated). Such a risk is of relevance in elderly because accumulating data suggest that persistent infection with Varicella-zoster virus (VZV; Arvin 1996), Epstein-Barr virus (EBV; Stowe et al. 2007) and particularly CMV (McVoy and Adler 1989) impacts upon the immune system in aging and may contribute to the immune risk phenotype (IRP), which predicts remaining longevity in the very elderly (Pawelec et al. 2005). Specific study on these aspects should be encouraged taking into account the possible relevant implications for public health.

3.4 Interrelationship Between Zinc and Selenium: Implications for Healthy Ageing

Dietary zinc and selenium are important nutritional factors for the immune response in protecting against the appearance of age-related diseases. The regulation of zinc ion bioavailability by selenium and selenoproteins has been recently investigated (Maret 2003). Zinc/thiolate coordination occurs in MT affecting the binding and release of zinc from MT. Zinc/thiolate cluster of MT can be oxidized by glutathion disulfide (GSSG) or other disulphides in order to release zinc. However, the efficiency of this chemical reaction seems very low even at high concentrations of GSSG in the absence of selenium. In contrast, the release of zinc from MT occur very rapidly following the addition of selenium compounds that has the capacity to form a catalytic selenol(ate), releases zinc (Maret 2003). The mechanism of the reaction was suggested to proceed through an activated selenenyl sulphide R-Se-S-G intermediate which, in turn, oxidizes the zinc-thiolate cluster of MT to form R-Se-S-MT with the concomitant release of zinc during the oxidation (Chen and Maret 2001). The selenol group is subsequently released by the attack of a nearby thiol group of MT that convert R-Se-S-MT into thionein generating a catalytic cycle of oxidative zinc release from MT. Other oxidized selenium compound, such as selenoxide and selenic acid may be directly reduced by MT through the formation of a R-Se-S-MT intermediates and the concomitant release of zinc, followed by the formation of an inter- or intramolecular disulfide bond (Chen and Maret 2001; Jacob et al. 1999; Klotz et al. 2003).

Selenium compounds also catalyze the release of zinc from MT in peroxidation and thiol/disulfide-interchange reactions. In presence of t-butylhydroperoxide, GPx catalyses the MT oxidation with subsequent zinc release, suggesting that MT may serve as reducing agents for GPx (or at least some GPx isoforms) in alternative to GSH (Jacob et al. 1999).Therefore, the assessment of zinc ion bioavailability, MT and selenium concentrations could represent useful tools for studying the physiology of successful ageing. Indeed, a recent study shows that 84.4% of the 'healthy' nonagenarian/centenarians display both zinc and selenium levels equal or greater than the lowest values in the elderly (Savarino et al. 2001). Moreover, healthy nonagenarians display low MT, good zinc ion bioavailability (Mocchegiani et al. 2002a) and satisfactory GPx activity (Mecocci et al. 2000). These findings suggest that an adequate zinc and selenium content in cells and tissues are crucial to achieve health ageing and longevity. In this context, Girodon et al. (1999) determined the effects of a long-term (for 2 years) daily supplementation with zinc (20 mg) plus selenium (100 μg) on immunity and the incidence of infections in a large number (n.725) institutionalized elderly people (> 65 years). The main results of the study were: (1) selenium deficient patients decreased from about 80% to 5–10% in the selenium supplemented group after 6 months of supplementation with respect to placebo group; (2) antibody titres after influenza vaccine were higher in groups that receive trace elements; (3) trace element supplemented patients were those who remained most free of respiratory tract infections than placebo group. These findings suggest that low dose supplementation of zinc and selenium provides significant improvement in elderly patients by increasing the humoral response after vaccination and decreased influenza compliances (respiratory tract infections) with thus possible achievement of health longevity.

4 Conclusions and Future Remarks

Beneficial effects obtained by zinc and selenium supplementation alone or associated on immune response and at clinical level are summarized in Table 1. Therefore, even if some controversial finding exists on the "real" necessity of micronutrient supplementation (Dangouret al.2004), the huge amount of data reported associated to observational data, clearly suggests that zinc and selenium play a pivotal role for immunosenescence in order to achieve healthy ageing and longevity. However, zinc seems to plays the major role because some biochemical mechanisms involved in the action of selenium are under the control of zinc ion bioavailability, which in turn is affected by MT and ZnT expression. One of the most relevant biochemical pathways is the release of zinc by MT through interactions with GPx and intracellular disulphides. However, some points require further investigations. First of all, the reason of a possible limited zinc release in ageing and the biochemical mechanism involved, in particular addressing NO-related intracellular pathways. Such an investigation is relevant taking into account the double face of NO action: or as antioxidant or as inducer of cell death (Colasanti and Suzuki 2000). Although useful tools are now available, such

Table 1 Possible causes of zinc and selenium deficiency in ageing and the main positive immune and clinical/biochemical effects of the related supplementation in experimental animals, in elderly and in syndrome of premature ageing (Down's syndrome)

Micronutrients	Possible causes of micronutrient deficiency in ageing	Immune and clinical/biochemical positive effects of micronutrient/s supplementation
Zinc	Frequent deficiency due to low dietary intake, enhanced urinary excretion, intestinal malabsorption. A limited zinc release from MT has been also proposed.	1. Enhanced NK cell cytotoxicity, cell-mediated immune response and thymulin activity; increased IFN-γ production, reduced levels of activated T helper cells; improved response to skin-test antigens and taste acuity 2. Lowering of plasma lipid peroxide levels 3. Restoration of TH1/TH2 paradigm 4. Increased ZnT1 and ZnT expression in lymphocytes 5. Reduced incidence of infection relapses in elderly, old infected patients and Down's syndrome subjects 6. Marginal effects on copper levels 7. Increased rate of survival in old, nude and thymectomized mice 8. Preservation of liver NKT γδ cells in old mice 9. Preservation of liver NKT cell cytotoxicity in old mice 10. Inducing apoptosis of only damaged cells in presence of high oxidative stress
Selenium	Decline with age mainly due to intestinal malabsorption	1. Increased lymphocyte mitogen response 2. Increased IL-2 receptor expression 3. Increased neutrophil function 4. Increased NK cell cytotoxicity 5. Decreased lung, colorectal and prostate cancer incidence 6. Lowering of cancer mortality 7. Less incidence of cardiovascular diseases 8. Decreased virulence of ECV, CBV and CMV
Selenium plus zinc		1. Improvements of antibody titres after influenza vaccination 2. Decreased influenza compliances (respiratory tract infections)

as NO donors and zinc fluorescent probes (zinpyr-1 and fluozin-3), in order to test the capacity of the cells in the zinc release by MT, the quantity of labile intracellular zinc in old age remains to be furtherly explored. This last point is also crucial because a fine modulation of intracellular labile zinc is fundamental in order to avoid an excessive zinc release by MT that can result toxic for the cell with subsequent cell-death. Moreover, the association of these studies with the role played by ZnT in ageing may give a more exhaustive picture of the role played by zinc in ageing. The results may

form a rationale to select old individuals who effectively need zinc supplementation because zinc, in a some extent, may be also toxic for the immune system leading to a further worsening of the already dysfunctional immune functions in ageing. In fact, many clinical trials of zinc supplementation in elderly report contradictory data on the benefit of zinc supplementation upon the immune functions. Thus, it is necessary to have many useful tools to screen real zinc-deficient old subjects. Among these tools, the genetic screening for some polymorphisms of MT, such as MT1A, might constitute a useful additional value in screening old subjects healthy ageing and longevity. Indeed, old subjects noncarriers of the C allele for MT1A +647 polymorphism display a better preservation of intracellular zinc homeostasis at advanced age, less inflammation, and are predisposed to the longevity with respect to old subjects carrying the C allele for the same MT polymorphism. This finding further suggests that only a certain number of old subjects are prone to zinc supplementation, and not all old population. In the case herein reported, a simple genotype screening might be useful to check the old subjects who should more frequently assess their zinc status for a possible zinc supplementation. In this context, genetic studies and the effect of zinc supplementation exclusively in old subjects with determinate polymorphisms for MT and IL-6 are studied in ZINCAGE project (www.zincage.org) funded by European Commission (EC) in FP6. Another project funded by EC in FP5 (ZENITH) confirms the presence of defects in zinc status and immune response in elderly. However, the biology of zinc is very complex and further studies are necessary in ageing especially addressed to the zinc-binding proteins strictly related to the inflammation and oxidative stress because both these conditions are the basis for the onset of a possible zinc dyshomeostasis in elderly (Mocchegiani et al. 2006).

With regard to selenium, the mechanisms of action of Se through selenoproteins against oxidative damage have been clear established, even if some aspects at genetic level especially regarding to the glutatione peroxidases require further studies. Indeed, while on one hand the genomic sequence of all GPxs isoforms has been established, the evolutionary reasons of an incorrect splicing of the selenium-independent GPx5 in humans is still to investigate. Anyway, selenium through selenoproteins has a wide range of action affecting the antioxidant system, the thyroid hormones turnover and the immune functions with a special focus on innate immune response. On the other hand, a correct thyroid hormone turnover affects the immune performances (Mocchegiani et al. 2006). As such, selenium treatment has been performed in various pathologies characterized by selenium deficiency, high oxidative stress and impaired immune function, such as cancer, infections, cardiovascular diseases as well as ageing. In this context, the more intriguing finding is the discovery that selenium deficiency in the diet or in soil is implicated in the mutation of a normally avirulent B3 coxsackievirus (CBV3/0) into a virulent virus (CBV3/20) by inducing changes in viral genoma. Moreover, a marginal selenium deficiency (1.10 μmol/L) causes a higher rate of mortality (by cancer) in old people with respect to old individuals with baseline selenium values (1.25 μmol/L). Therefore from the review data herein reported, zinc and selenium in the daily diet during ageing may be relevant in order to preserve immune and antioxidant functions, which can lead to healthy ageing and longevity. Alternatively, a

combined oral supplementation of these micronutrients can be recommended taking into account the beneficial effects of zinc and selenium in improving the humoral immune response in old vaccinated individuals (Duchateau et al. 2004; Girodon et al. 1999). However, the gap between the estimated average requirement of zinc and the upper limit of safe intake is relatively narrow, because excessive zinc may be toxic (Fosmire 1990). Concerning to selenium, even if few reported cases have been associated with an excessive intake of selenium, it has to be taken into account that the Institute of Medicine of the National Academy of Sciences has set a tolerable upper intake level for selenium at 400 micrograms per day for adults to prevent the risk of developing selenosis (Johnson et al. 2003). Therefore, supplementation with zinc and selenium can be recommended in old people who effectively need zinc and/or selenium supplementation after a careful evaluation of the "zinc/selenium status" through plasma measurement, clinical features and possibly evaluating the intracellular content of zinc and selenium. The usefulness of MT polymorphisms in identifying subjects at risk for zinc deficiency might be an additional tool. As such, the impaired immune functions in elderly, through these two trace elements, may be restored with subsequent healthy ageing and longevity.

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Lipids

Immunomoduation by Polyunsaturated Fatty Acids: Impact on T-cell Functions and Signaling

Maximilian Zeyda and Thomas M. Stulnig

Contents

Abbreviations

AA	arachidonic acid
APC	antigen-presenting cell
COX	cyclooxygenase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ERK	extracellular signal-regulated kinase
GPI	glycosylinositolphasphatidyl
ICAM	intercellular adhesion molecule
IFN	interferon
IL-2(R)	interleukin-2 (receptor)
IP_3	inositol(1,4,5)-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun NH2-terminal kinase
LAT	linker for activation of T cells

M. Zeyda (✉) · T. M. Stulnig
Department of Internal Medicine III
Medical University of Vienna
Währinger Gürtel 18-20, A-1090 Vienna, Austria
Tel.: +43 1 40400 4336; +43 1 40400 4368
Fax: +43 1 40400 7790
E-mail: maximilian.zeyda@meduniwien.ac.at; thomas.stulnig@meduniwien.ac.at

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LFA	leukocyte functional antigen
LT	leukotrienes
LXR	liver X receptors
MAPK	mitogen-activated protein kinases
MHC	major histocompatibility complex
PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
PG	prostaglandin
PIP_2	phosphatidylinositol(4,5)-bisphosphate
PKC	protein kinase C
PLC/D	phospholipase C/D
PPAR	peroxisome-proliferator-activated receptor
PUFA	polyunsaturated fatty acid
SMAC	supramolecular activation cluster
STAT	signal transducer and activators of transcription
TCR	T cell antigen receptor
TX	thromboxane

Abstract: Long chain polyunsaturated fatty acids (PUFAs) are well-known for their beneficial immunomodulatory effects in a variety of autoimmune and inflammatory disorders. The underlying molecular mechanisms are manifold, but are still elusive to a large extent. Several cell types are target of PUFA action. In this chapter, the effects of PUFAs on T-cell activation and function are discussed. PUFAs directly affect T-cell signaling and thus activation as well as T-cell interactions with antigen-presenting cells (APCs). The mainstream of publications in the field describes alterations of the lateral membrane organization as crucial for PUFA action on T-cells. Therefore, this chapter includes a brief overview over the current understanding of membrane microdomains, so-called "lipid rafts," and their role in T-cell signal transduction.

Keywords: Immunological synapse • Lipid rafts • Omega-3 fatty acids • Signal transduction • T-lymphocytes

1 Introduction

Long chain polyunsaturated fatty acids (PUFAs) are well-known for their beneficial effects in a variety of autoimmune and inflammatory disorders. The underlying molecular mechanisms are manifold but are still elusive to a large extent (Stulnig 2003). Here, we discuss the effects of PUFAs on T-cell activation by directly affecting T-cell signaling as well as T-cell interactions with antigen-presenting cells (APCs). The mainstream of publications in the field describes alterations of the lateral membrane organization as crucial for PUFA action on T-cells. Therefore, this chapter includes a brief overview over the current understanding of "lipid rafts" and their role in T-cell signal transduction. Figure 1 gives a basic overview over these mechanisms as discussed in this chapter. PUFA-mediated alterations and their relation to immunosenescence are discussed elsewhere in this book.

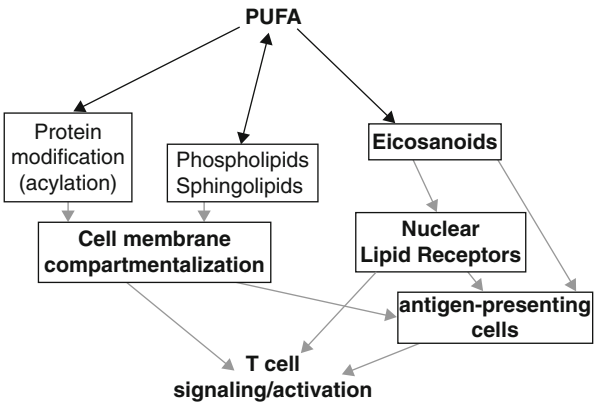

Fig. 1 Overview on mechanisms underlying PUFA effects on T-cell signaling and activation. Black arrows indicate metabolic conversions, gray arrows indicate possible effects on the designated items. For details see main text

1.1 PUFAs–a Short Introduction

Vertebrate animals lack desaturases that introduce double bonds methyl-terminal of Δ9 of the fatty acid chain. Therefore, fatty acids containing double bonds at the n-6 or n-3 position (counted from the methyl terminus) such as linoleic acid (18:2 (n-6)) and α-linolenic acid (18:3 (n-3)) are essential to them. The mentioned C18 essential fatty acids can undergo a series of elongations and desaturation steps that result in the formation of longer-chain n-6 and n-3 PUFAs but with varying efficiency. Most prominent long-chain PUFAs are arachidonic acid (20:4 (n-6), AA), eicosapentaenoic acid (20:5 (n-3), EPA) and docosahexaenoic acid (22:6 (n-3), DHA; Jump 2002). The most common dietary sources of n-6 PUFAs are corn, safflower, soybean, and sunflower oils. Sources for n-3 PUFAs are green leafy vegetables, walnuts, and rapeseed and flaxseed oils, but most of the long chain n-3 PUFAs are obtained directly from dietary intake of marine fish oils (Burdge and Calder 2005; Calder 2002). The observation that populations with high marine fish consumption, have a very low incidence of inflammatory and autoimmune disorders provided a basis for the hypothesis that n-3 PUFA possess immunoregulatory and antiinflammatory activities (Calder 1998).

1.2 PUFAs as Immunomodulatory Agents

PUFA effects are often modest in clinical studies and the results are not unequivocal. n-3 PUFAs have been shown to evoke clinically significant beneficial effects in patients with chronic inflammatory diseases such as Crohn's disease (Belluzzi et al. 1996), atherosclerosis (Thies et al. 2003; Zampolli et al. 2006), colitis (Mills et al. 2005), graft-versus-host disease (Takatsuka et al. 2002), psoriasis (Mayser

et al. 2002a), atopic dermatitis (Mayser et al. 2002b), multiple sclerosis (Gallai et al. 1995), asthma (Broughton et al. 1997), and systemic lupus erythematosus (Leiba et al. 2001). Probably the best evidence of PUFA effects is available for treatment of patients with rheumatoid arthritis (Fortin et al. 1995; Stamp et al. 2005). In addition to reducing morning stiffness and the number of tender joints, n-3 PUFA may be beneficial for rheumatoid arthritis patients since it decreases the need for antiinflammatory drugs (Calder 2006). Most strikingly, dietary supplementation with n-3 PUFA leads to a statistically significant reduction of fatal cardiovascular events in patients with prior myocardial infarction (GISSI Study Group 1999). PUFA effects have also described for other clinical issues related to dysregulated inflammation such as aging (Pepe 2005; SanGiovanni and Chew 2005; Yehuda et al. 2002) and insulin resistance (Suresh and Das 2006; Todoric et al. 2006; Winzell et al. 2006; Xiao et al. 2006). Possible anticancer properties of PUFAs are discussed controversially (Chapkin et al. 2007; MacLean et al. 2006).

1.3 Cellular Targets of PUFA Effects

Immunomodulatory effects of PUFAs, in particular n-3 PUFAs, in animal studies and different cell types in culture are well accepted (Calder 2006; K Fritsche 2006; Stulnig 2003). PUFAs inhibit monocyte/macrophage functions including cytokine production, phagocytosis, and expression of T-cell stimulatory molecules (Calder et al. 1990; Hughes et al. 1996; Lokesh et al. 1990). Furthermore, natural killer (NK) cell activity has been shown to be affected by PUFA (Meydani et al. 1988). On the other hand, PUFAs (n-6 and n-3) have been shown to induce a respiratory response and degranulation of cord blood neutrophils possibly counteracting anti-inflammatory actions (Ferrante et al. 1996). As a basis for further considerations in this chapter, it has been known for several years that PUFAs potently affect T-cells by inhibiting proliferation, surface activation marker expression, and cytokine production (Chapkin et al. 2002; Costabile et al. 2001; Fowler et al. 1993; Meydani et al. 1991; Pompos and Fritsche 2002; P. Zhang et al. 2006; Zurier et al. 1999).

1.4 General Mechanisms of Immunomodulatory PUFA Effects

PUFAs are precursors of immunologically active lipid mediators, i.e. eicosanoid messenger molecules such as prostaglandins (PG), leukotrienes (LTs) and thromboxanes (TXs). LTs and TXs are usually derived from AA that is liberated from membrane phospholipids by phospholipase A. Metabolism of AA by cyclooxygenases (COX) leads to generation of PG and TX of the 2-series, whereas metabolisation via 5-lipoxygenases gives rise to, e.g., LT of the 4-series. PUFA of the n-3 series interfere with the biosynthesis of AA-derived molecules and by themselves

give rise to chemically different mediator molecules. When EPA is metabolized instead of AA by COX, PG and TX of the 3-series are produced that exert attenuated or partially different biological effects (Calder 2002; Calder et al. 2002). Moreover, although the affinity of COX for EPA is low, EPA inhibits COX activity, in particular COX-1 activity, for AA oxygenation (Wada et al. 2007). In addition to directly interfering with enzymes of eicosanoid synthesis, PUFA can also affect protein levels of involved enzymes by altering gene expression as shown for COX-2 in monocytes (JY Lee et al. 2003a). Though n-6 and n-3 PUFA differently affect eicosanoid synthesis, the functional outcome of these changes with respect to immunomodulation are often not predictable. For instance, AA, but not EPA has been shown to inhibit IL-2-induced T-cell proliferation in vitro (Santoli et al. 1990). Moreover, in vivo interactions of the generated messenger molecules are hardly predictable. Differences of in vitro and in vivo eicosanoid production may occur (Knapp et al. 1986; Saito et al. 1997) and species differences in eicosanoid effects, as shown, e.g., in humans compared to rats (Morita et al. 1983). Hence, extrapolations of in vitro data to the in vivo situation have turned out to be extremely difficult.

Recent research has characterized endogenous mediators of resolution, the actively regulated program of returning from inflammation to a healthy state (Gilroy et al. 2004). These resolving lipid mediators, named resolvins and protectins, are synthesized in several enzymatic steps from EPA, DHA, and also n-6 AA (Serhan 2004, 2007) additionally disproving the paradigm that n-3 PUFAs act via simple replacement of n-6-derived inflammatory mediators. The contribution of these novel classes of PUFA-derived lipid mediators to the beneficial and antiinflammatory effects remains to be elucidated, but since they potently drive the program of resolution in nanomolar concentrations (Bannenberg et al. 2005; Schwab et al. 2007), the therapeutic potential of these lipid mediators appears promising.

Another principal mechanism for modulation of immune responses by PUFAs is through direct alteration of gene expression by binding and activation nuclear receptors, i.e. ligand-binding transcription factors. Peroxisome-proliferator-activated receptor (PPAR)γ preferentially binds a variety of PUFA and their derivatives and has been shown to be involved in lymphocyte activation and macrophage differentiation (Clark et al. 2000; Marx et al. 1998; Yang et al. 2000). Activation of PPARγ could be a mechanism of PUFA-mediated immunomodulation also by directly affecting T-cells (Clark et al. 2000; Deckelbaum et al. 2006; Yang et al. 2000). However, PPARγ is also activated by much more abundant monounsaturated fatty acids. In addition to PPARγ, PPARα and PPARδ can bind fatty acids, but with even less specificity for PUFA. Liver X receptors (LXR) α and β are inhibited by monounsaturated and PUFAs (Desvergne and Wahli 1999). Retinoid X receptors, the heterodimer partner for a variety of nuclear receptors including those mentioned above, is activated by DHA with some selectivity of AA (de Urquiza et al. 2000; Lengqvist et al. 2004). However, nuclear receptors generally lack adequate specificity for PUFA to explain their immunomodulatory effects. Moreover, suppressive effects in vitro have mostly been found with rather unselective nuclear receptor ligands so that the impact of nuclear receptors in general and PPARγ in particular on PUFA-mediated immunomodulation is doubtful (Jump 2002; Stulnig and Zeyda 2004).

2 Mechanisms of PUFA Effects on T-cell Signaling

2.1 T-cell Signal Transduction–The Conventional Concept

Activation of T-cells requires stimulation of the T-cell antigen receptor (TCR)/CD3 complex and costimulatory receptors. Triggering CD3 leads to rapid autophosphorylation of Src-family protein tyrosine kinases and phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the CD3 complex, thereby facilitating SH2 domain-mediated binding of other signaling molecules such as Syk-family kinases ZAP-70 and Syk (Kane et al. 2000; Wange and Samelson 1996). ZAP-70 associates with phosphorylated CD3ζ-ITAMs and is subsequently activated by Lck. Following phosphorylation by ZAP-70, the central adaptor protein linker for activation of T-cells (LAT) recruits phospholipase C (PLC)γ to the cell membrane (WG Zhang et al. 1998a). PLCγ1 is also activated by ZAP-70 to liberate inositol[1,4,5]-trisphosphate (IP_3) from the plasma membrane lipid phosphatidylinositol[4,5]-bisphosphate (PIP_2) thereby eliciting an increase in cytoplasmic calcium concentration, a key event for promoting downstream activation (Berridge et al. 2000). Activation of further signaling mediators is partially dependent on costimulatory signals that are triggered via costimulatory cell surface receptors such as CD28 (Schwartz 1992). In consequence to early protein phosphorylation steps and calcium response, mitogen-activated protein kinases (MAPKs) are activated by phosphorylation. The three major families of MAPKs, extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), and p38 MAPK, are regulated by distinct but cross-talking signaling cascades (Garrington and Johnson 1999). Such signals culminate in the activation of transcription factors such as NF-AT, AP-1, and NF-κB (Baeuerle and Henkel 1994; Karin et al. 1997; Masuda et al. 1998). These transcription factors bind recognition sites within promoter sequences to induce transcription of cytokines including interleukin-2 (IL-2), the major T-lymphocyte growth factor (Cantrell 2002). Thus, T-cell stimulation leads to interleukin production and proliferation, thereby promoting the adaptive immune response.

2.2 PUFA Effects on T-cell Signaling and Activation

The most upstream T-cell signaling events affected by PUFA treatment are phosphorylation of LAT and PLCγ and calcium signaling (Chow et al. 1991; Stulnig et al. 1998; Zeyda et al. 2002). Further downstream at the level of MAPK, PUFA highly selectively inhibit JNK phosphorylation and activation, whereas phosphorylation of p38 MAPK and ERK-1 and-2 remain essentially unaltered by PUFA treatment of Jurkat and peripheral blood T-cells (Zeyda et al. 2003). Analyses of transcription factor activation revealed an inhibition of NF-AT activity, while activation of AP-1 and NF-κB are not affected by PUFA-treatment. However, at least in Jurkat cells,

an involvement of distinct protein kinase C (PKC) isoforms and NF-κB has been reported (Denys et al. 2005), and PUFA-mediated inhibition of NF-κB activation has been confirmed in rats (J. Wang et al. 2007b).

On level of cytokine expression, PUFA were found to inhibit IL-2 and IL-13, but not interferon (IFN)-γ, IL-4, IL-9, or IL-10 further indicating selectivity of PUFA effects rather than general T-cell inhibition (Zeyda et al. 2003). Half-maximal effects for inhibition of IL-2 occurred at about 5 μM suggesting significance also for in vivo PUFA treatment. In addition, expression of the cell surface activation markers CD25, but not of CD69 was strikingly inhibited by PUFA treatment. Thus PUFA inhibit T-cell downstream signaling in a highly selective manner (Zeyda et al. 2003).

In contrast to these in vitro data, in vivo data on PUFA effects on T-cell driven immune esponses have not shown such high selectivity in inhibition but more general effects e.g., including reduction of serum concentrations of IFN-γ (KL Fritsche et al. 2000; J Wang et al. 2007b). These differences could be due to different ways of T-cell activation in vivo compared to in vitro. In vivo, T helper cells that drive immune responses are stimulated via APCs. For efficient T-cell stimulation, the contact site between T-cell and APC requires a complex organization known as "immunological synapse." A study shows that formation of the immunological synapse is altered when T-cells have been treated with PUFA (Geyeregger et al. 2005). Such alterations (discussed in detail below) could lead to diminished activation of pathways that are not intrinsically altered in PUFA-treated T-cells when stimulated via antibodies to cell surface receptors. Hence PUFA-mediated alterations in IS formation could underlie the more general deficiency in T-cell activation found in vivo. On the other hand a recent in vivo study suggests increased expression of the downregulatory coreceptor CTLA-4 to be responsible for the PUFA-mediated block in CD4+ T-cell activation (Ly et al. 2006). Hence, PUFA effects and mechanisms are manifold and an estimation of the relative contribution of different mechanisms to PUFA-mediated alterations of T-cell activation in vivo is extremely difficult. According to the availability of convincing experimental data, this book chapter focuses on PUFA-mediated alterations of the cell membrane and their functional consequences.

2.3 Lipid Rafts and Their Role of in T-cell Signaling

The lipid raft model is based on the observation that cholesterol and sphingolipids are not distributed evenly in the plasma membrane as suggested by the classical fluid mosaic model (Singer and Nicolson 1972), but rather assemble to microdomains ("rafts") in an so-called "liquid ordered" state that float within the rest of the membrane (DA Brown and Rose 1992; RE Brown 1998; Rietveld and Simons 1998). The formation of lipid domains within the cell membrane facilitates a spatial sequestration of membrane proteins. The most common biochemical method to analyze lipid rafts is based on the partial insolubility of membranes in nonionic

detergents such as Triton X-100 at 4°C. As a consequence, when such membrane or cell lysates are subjected to density gradient ultracentrifugation, the detergent-insoluble membranes float to low density fractions and can be separated from soluble and nonmembrane fractions. Together with lipid components, membrane proteins are also separated into detergent soluble and insoluble fractions. Proteins found in detergent-insoluble fractions fulfill specific roles in cellular processes, particularly in cell signaling, suggesting a functional role of rafts in these processes (Simons and Ikonen 1997). Of note, the nature and even the existence of lipid rafts is a matter of debate (Munro 2003). Novel experimental data of determination of single molecule movements (Dietrich et al. 2002; Drbal et al. 2007; Fujiwara et al. 2002; Kusumi et al. 2005; Kusumi and Suzuki 2005; Schutz et al. 2000; Wieser et al. 2007) as well as the role of protein protein interactions have to be integrated into the model of membrane compartmentalization (Zeyda and Stulnig 2006). But in spite of modifications to be made on the concept of lipid rafts, the lipid raft model definitely helpful for the understanding of T-cell signal transduction as well as PUFA effects.

Several mechanisms target proteins to detergent insoluble membrane domains. For instance, glycosylinositolphasphatidyl (GPI)-anchored proteins are generally targeted to lipid rafts and have frequently been used as lipid raft markers (Cinek and Horejsi 1992). GPI anchors consist of a phosphatidylinositol typically containing two long-chain acyl moieties (Roberts et al. 1988) that insert into the exoplasmic leaflet of the membrane and a head group linked via an amide bond to the C-terminal residue of the protein, which usually has no other direct connection to the membrane. Another type of lipid modification that targets proteins to lipid rafts is acylation such as myristoylation and palmitoylation (Shenoy-Scaria et al. 1993; Zacharias et al. 2002). In general, two saturated acyl moieties target proteins to lipid rafts, independent of whether a protein spans the membrane or is linked to the membrane merely by the lipid (Moffett et al. 2000). Prenylated proteins do not generally associate with rafts. Ras proteins may be prenylated and additionally palmitoylated allowing differential targeting to raft and nonraft fractions due to variations of lipid modifications (Melkonian et al. 1999; Roy et al. 2005). Of note, acylated proteins are attached to the inner leaflet of the cell membrane, in contrast to GPI-anchored proteins, which are located at the cell surface.

Targeting specific proteins to rafts enables a spatial organization of membrane proteins that preferentially reside in, or are excluded from rafts. In consequence, confined zones with specialized functions due to particular protein composition are created. Moreover, a fine-tuning of protein affinity for distinct lipid environment, possibly supported by protein-protein interactions (McConville and Menon 2000; Shogomori et al. 2005) may be a basis for lipid raft heterogeneity. Raft heterogeneity is indicated by experimental data and could not only include the existence of different subsets of rafts, but also zones within one raft (McCabe and Berthiaume 2001).

Strikingly, many cytosolic proteins that are linked to the membrane by lipid anchors are crucial signaling mediators. Accordingly, Src family kinases such as Lck and Fyn (Arreaza et al. 1994) and GTPases such as H-Ras (Prior and Hancock 2001) are enriched in rafts. In contrast, most transmembrane proteins, e.g., the phosphatase CD45, are generally excluded from rafts unless they are acylated such as the TCR

coreceptors CD4 and CD8b (Cerny et al. 1996) and adaptor proteins including LAT (WG Zhang et al. 1998b) and phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG, also named Cbp; Brdicka et al. 2000; Kawabuchi et al. 2000), both of which are important molecules of the T-cell signaling machinery.

Alike most transmembrane proteins, components of the TCR, i.e., the clonally specific heterodimer noncovalently associated with the invariant CD3 adapter complex, are found outside of lipid rafts and thus spatially separated from raft resident molecules that mediate the cytosolic signaling processes. The decisive clue for the common model how ligand binding to TCR mediates intracellular signaling was the observation that, depending on the Src family kinase activity, phosphorylated CD3 ζ chains can be found within lipid rafts upon TCR stimulation together with phosphorylation-activated ZAP-70 (Montixi et al. 1998). Hence, it appeared plausible that upon ligand binding the TCR/CD3 complex is recruited to membrane rafts and thus moves from an environment containing inhibitory phosphatases such as CD45 to confined zones with enhanced signaling activity. However, the CD45 phosphatase has recently been found to predominantly activate Lck activity by inducing conformational changes, and small amounts of CD45 are targeted to rafts as assessed by Triton X-100 extraction. An interpretation of the role of CD45 is to keep Lck activation balanced by counteracting the Lck-deactivating kinase Csk (Davidson et al. 2003; Irles et al. 2003). Other findings point towards another interpretation, namely that raft-excluded CD45 positively regulates T-cell activation (M Zhang et al. 2005).

The notion of lipid rafts as functional signaling microdomains was corroborated by experimental disintegration of lipid rafts by cholesterol depletion with methyl-β-cyclodextrin and polyene antifungal agents filipin and nystatin, which impair TCR-mediated signaling (Xavier et al. 1998). Since only phosphorylated CD3ζ is recruited to lipid rafts and CD3 phosphorylation is supposed to occur only inside rafts according to the raft localization of Lck, it remained unclear which mechanisms could mediate the induced translocation of TCR components to rafts. A possible solution for this problem may be the finding that TCR components can be recovered from isolated rafts constitutively and independent of Src-family kinase activity when raft were isolated at physiological temperature using Brij 98, a detergent characterized by its relatively bulky polyoxyethylene headgroup and monounsaturated ether moieties (Drevot et al. 2002). According to this finding, CD3 tyrosine phosphorylation has been suggested to be initiated upon ligand-induced conformational changes of the TCR complex rather than by translocation of the TCR into lipid rafts (Drevot et al. 2002).

Lipid rafts are not only involved in the initiation of the most upstream signaling events, but also stabilize and amplify existing signals. This function is enabled by aggregation of rafts to larger complexes accompanied by further recruitment of important signaling mediators such as LAT and associated molecules (Janes et al. 1999) as well as TCR components (Valensin et al. 2002). These raft complexes were given the sounding term “signaling platforms” (Hoessli et al. 2000). Importantly, rafts and filamentous actin (F-actin) were found to colocalize (Harder and Simons 1999) whereby the actin cytoskeleton drives the lipid raft aggregation (Rodgers and Zavzavadjian 2001; Valensin et al. 2002; Villalba et al. 2001). Moreover, the cytoskeleton-driven movement of membrane molecules leads to an

increase of the overall amplitude and duration of T-cell signaling (Wulfing and Davis 1998). Such molecular movements induce an accumulation of molecules at the interface of the T-cell and the APC (Wulfing and Davis 1998), underlying the concept of the immunological synapse (*See* below). The most prominent TCR-induced signaling pathway driving the activation of the cytoskeleton functions via Vav, a 95 kDa nucleotide exchange factor that is activated by tyrosine phosphorylation (Crespo et al. 1997; Fischer et al. 1998) and associated to the LAT signalosome (Clements et al. 1999). Conversely to the function of the cytoskeleton in driving lipid raft aggregation, rafts are necessary for activation of the actin skeleton and GPI-anchored proteins provide costimulatory signals leading to reorganization of the cytoskeleton (Moran and Miceli 1998).

2.4 PUFA-Mediated Alterations of Lipid Rafts and Consequences in Signaling

Upon dietary intake PUFAs are distributed throughout the body like other fatty acids and can be taken up by basically every cell type (Jump 2002). Dietary consumption of PUFA can significantly increase the relative PUFA content of cell membranes. This is particularly true for n-3 PUFAs whose abundance in western diets and T-cell membranes is generally rather low (Fowler et al. 1993). PUFAs are predominately esterified to the *sn*-2 position of phosphatidylcholine and phosphatidylethanolamine phospholipids. Unsaturated, particularly polyunsaturated acyl chains, do not pack well with cholesterol molecules. Therefore, PUFAs avoid liquid ordered phases (i.e., lipid rafts) in lipid bilayers, which may contribute to phase separation (Shaikh et al. 2003). Uptake of PUFAs by T-cells leads to their incorporation into detergent-resistant domains as shown in vitro by T-cell treatment with PUFAs (Stulnig et al. 2001) as well as in vivo by fish oil feeding of mice (Fan et al. 2003; Switzer et al. 2003). PUFA incorporation into raft lipids not only alters their unsaturation index but most probably also their biophysical properties such as the tightness of lipid packaging.

Importantly, PUFA-mediated alterations of raft lipid composition are associated with altered protein composition. Treatment of Jurkat T-cells with linoleic acid, EPA, and DHA leads to a potent reduction of lipid raft localization of the Src-family kinases Lck and Fyn (Stulnig et al. 1998). Strikingly, these alterations in raft protein content directly correlated with the observed inhibition of calcium signaling (Stulnig et al. 1998). Moreover, in Jurkat as well as peripheral blood T-cells, PUFAs displace LAT from rafts (Zeyda et al. 2002). For this effect, two principal but not mutually exclusive mechanistic explanations exist: palmitoyl transferases are nonselective and can covalently attach PUFAs to proteins. Accordingly, Src family kinases may be acylated with PUFAs such as EPA and AA when these are overabundantly present leading to diminished targeting of these proteins to lipid rafts (Liang et al. 2001; Webb et al. 2000). On the other hand, the described alterations of raft lipid composition may alter the affinity of palmitoylated proteins to these microdomains. Notably, PUFA treatment leads to enrichment of typical inner leaflet phospholipids

such as phosphatidylethanolamine with PUFAs (Stulnig et al. 2001). The occurrence of [^{3}H]-palmitoyl labeled proteins in nonraft fractions strongly suggests, that PUFA alterations of raft lipid rather than PUFA acylation of proteins is the predominant mechanism of PUFA-mediated displacement of acylated proteins from rafts (Stulnig et al. 2001).

Irrespective of the relative contribution of the mentioned principal mechanisms for the alterations of the lipid raft protein composition, the adapter LAT has been shown to be a central target of PUFA-mediated inhibition of early T-cell signal transduction (Zeyda et al. 2002). As mentioned above, PUFA treatment of T-cells inhibits TCR-induced phosphorylation of LAT and PLCγ, whereas tyrosine phosphorylation of CD3, binding of ZAP-70, and subsequent phosphorylation of ZAP-70 remains unaffected. Strikingly, a genetically modified chimeric LAT protein that contained the transmembrane and extracellular domain of PAG, which remained within rafts upon PUFA treatment, restored tyrosine phosphorylation of PLCγ and calcium response after PUFA treatment. Notably, other signaling proteins, e.g., Lck, are still displaced from rafts in PAG-LAT reconstituted T-cells demonstrating the crucial role of LAT raft localization for efficient transduction of the TCR signal (Zeyda et al. 2002). In looking for a suitable partner molecule for LAT, PAG's localization in lipid rafts turned out to be resistant to PUFA-mediated alterations. The reason for this special behaviour is still unknown. However, elucidating the underlying molecular characteristics could open a new view on the true mechanisms of PUFA-medited lipid raft alterations and/or on mechanisms by which proteins are targeted to lipid rafts. In a further study it has been shown that the displacement of LAT from lipid rafts is also directly responsible for diminished tyrosine phosphorylation of Vav (Geyeregger et al. 2005). This effect probably causes diminished cytoskeletal activation and hence protein translocations that are necessary for T-cell activation by APCs, as discussed below. A similar, but different mechanism of PUFA action was described in T-cells of fish oil-DHA-fed mice, which show a reduced recruitment PKCθ to lipid rafts upon stimulation and accordingly diminished activation and effector functions of PKCθ (Fan et al. 2004).

Another mechanism of PUFA action on T-cells that involves alterations of lipid rafts is derived from the finding in Jurkat Tcells that lipid raft disintegration activates phospholipase D (PLD), which negatively regulates cell proliferation (Diaz et al. 2005). Indeed, also DHA treatment of peripheral blood mononuclear cells, which inhibited concanavalin A-induced proliferation, shifted PLD to nonraft fraction where it was activated by ADP-ribosylation factor (Diaz et al. 2002). Additionally, PUFA-mediated lipid raft alterations could affect T-cell responses via activation-induced cell death, a form of apoptosis resulting from chronic antigen stimulation and necessray for the deletion of activated T-cells. Interestingly, n-3 PUFAs enhanced activation induced cell death (Switzer et al. 2003; Switzer et al. 2004a), which may be due to altered submembrane distribution of the Fas death receptor and components of the death-inducing signaling complex (Switzer et al. 2004b).

Lipid raft modifications by PUFAs not only affect the antigen/TCR-mediated activation of T-cells as discussed so far, but may also underlie inhibition of IL-2-induced proliferation and IL-2 receptor (IL-2R) signaling as shown for AA and

DHA, respectively (Li et al. 2005; Santoli et al. 1990). Activation of IL-2R leads to phosphorylation of Janus kinases, which activate signal transducer and activators of transcription (STAT) proteins. Activated STATs are then recruited to the nucleus, where they induce gene transcription. PUFA-mediated raft lipid alterations correlate with a reduction in IL-2Rα surface expression and displacement of IL-2Rα, β, and γ_c chains as well as STAT5a and b from lipid rafts (Li et al. 2005). However, it should be mentioned that these results are not in line with a prior description of the raft/nonraft distribution of the components of IL-2R and proposed models for the role of lipid rafts in IL-2R signaling (Marmor and Julius 2001).

3 PUFA Effects on T-cell/APC Interactions

3.1 T-cell Stimulation by APC and the Immunological Synapse

T-cell activation via the TCR not only depends on mere ligation of the TCR and costimulatory receptors, but also on a complex interaction of T-cells with APCs. Due to some similarities to the neural synapse the interface between T-cell and APC has been named "immunological synapse" (Dustin and Colman 2002). When a T-cell encounters an APC, T-cell cytoskeletal, adhesion, and signaling proteins aggregate at the contact site to the APC, building "supramolecular activation clusters" (SMACs; Monks et al. 1998). SMACs are spatially and temporally organized structures crucial for controlling and balancing signals, the strength and nature of which depend on the abundance of antigen as well as on the type of APC (Huppa; KH Lee et al. 2003b). During synapse maturation, i.e., the complex relocalization of cell surface molecules that takes about 15 min, some proteins including the TCR complex accumulate at the center of the immunological synapse, named c-SMAC, whereas others such as the integrin leukocyte functional antigen (LFA)-1 become located at its periphery (*p*-SMAC; Monks et al. 1998; Montoya et al. 2002). Since the LFA/intercellular adhesion molecule (ICAM) binding pair requires a larger intercellular distance than a TCR/major histocompatibility complex (MHC) pair, the larger binding pairs in intercellular junction between T-cell and APC are displaced to the periphery whereas shorter ones are concentrated in its center. The phosphatase CD45 was found to segregate to the cSMACs at early stages of synapse formation and to be displaced to outer regions after synapse maturation later on (Freiberg et al. 2002). Also CD28 accumulates at the immunological synapse (Andres et al. 2004; Wetzel et al. 2002). Although results from theoretical studies have hypothesized that the immunological synapse could form in the absence of active, energy-expending processes (Qi et al. 2001), accumulation of molecules at the immunological synapse is an active process, driven by the cytoskeleton and depending on distinct signals provided by the APC (Andres et al. 2004; Villalba et al. 2001; Wetzel et al. 2002; Wulfing and Davis 1998; Wulfing et al. 2002).

Briefly summarizing the complex data reviewed in detail elsewhere (Zeyda and Stulnig 2006), the process of T-cell stimulation by APC could be described, very con-

cisely, as follows: In unstimulated T-cells the membrane is compartmentalized in a temporally and spatially very dynamic manner by lipid-based molecule interactions. Immediately after contact with an APC, costimulatory receptors including adhesion molecules, GPI-anchored receptors, CD28, and CD2, possibly together with stimulation of few TCR molecules mediate reorganization of the cell membrane by receptor-mediated raft stabilization, induction of tyrosine phosphorylation, and nucleation of large signaling protein complexes (signalosomes) leading to activation of the actin cytoskeleton and formation of an immature immunological synapse. Thereby, the signaling machinery located in distinct lipid rafts is translocated to the APC contact site and induce cell–cell adhesion providing an enviroment that facilites T-cell stimulation. Due to TCR stimulation, lipid raft aggregation occurs and LAT-nucleated signalosomes associate with TCR complexes, forming TCR/LAT signalosomes that induce further cytoskeletal reorganization and formation of a mature immunological synapse. Finally, activation signals are transduced to the nucleus to induce gene transcription and promote T-cell-mediated immune responses (Zeyda and Stulnig 2006).

3.2 PUFA Effects on Immunological Synapse and T-cell/APC Conjugate Formation

Disintegration of the immunological synapse results in diminshed T-cell activation (Huppa et al. 2003) and interference with synapse formation appears to be a potential mode of action of immunosuppressive and antiinflammatory chemokines (Bromley et al. 2000), in orally induced systemic immune hyporesponsiveness (Ise et al. 2005), and could represent a promising target for immunosuppressive and antirheumatic drugs (Zeyda et al. 2005a, 2007) including PUFA.

Consequently, our group thoroughly analyzed PUFA effects on immunological synapse formation (Geyeregger et al. 2005). PUFA treatment affects superantigen-induced formation of the mature (after 15 min of stimulation) but not immature (after 1min) immunological synapse, particularly by inhibiting the relocalization of adhesion (LFA-1), cytoskeletal (F-actin and talin) and signaling molecules (CD3 and LAT) with the exception of PKCθ. The selectivity for inhibition of the mature but not the immature IS was associated with diminished sustained (after 15 min of stimulation) but not early (after 1 min) phosphorylation of Vav in PUFA-treated T-cells (Geyeregger et al. 2005). Vav activity drives cytoskeletal rearrangements (Ardouin et al. 2003; Krawczyk and Penninger 2001) necessary for relocalization to the immunological synapse of, e.g., LFA-1, which is crucial for the formation of high affinity T-cell/APC interaction (Morgan et al. 2001). Accordingly, beyond qualitative changes in IS formation, PUFA treatment also markedly reduces the efficiency of conjugate formation between T-cells and APCs in an antigen-specific manner (Geyeregger et al. 2005). As a consequence, diminished conjugate and altered IS formation could extend PUFA defects in T-cell activation to signaling pathways that are not intrinsically altered in PUFA-treated T-cells as assessed by antibody-mediated T-cell stimulation. Accordingly, stimulated expression of CD69 that is unchanged in antibody-stimu-

lated T-cells is inhibited in PUFA-treated T-cells stimulated with antigen presented on APCs (Geyeregger et al. 2005). To investigate the underlying mechanisms of these effects, we focused on LAT due to its central role in lipid raft-controlled early T-cell signal transduction (Zeyda and Stulnig 2006) as well as in PUFA-mediated effects on T-cell signaling (Zeyda et al. 2002). We found that LAT displacement from lipid rafts underlies PUFA-mediated inhibition of CD3 capping, which is an active cytoskeletal-induced process mimicking molecule rearrangements during IS formation. CD3 capping in LAT-deficient cells reconstituted with chimeric LAT protein that retains lipid raft localization in presence of PUFA rrremained unaltered by PUFA treatment in contrast to cells reconstituted with wild-type LAT (Geyeregger et al. 2005). Hence, inhibition of Vav downstream signaling leading to defective immunological synapse formation probably originates from modification of T-cell lipid rafts resulting in displacement of LAT from rafts. Further evidence for this model is provided by the fact that PUFA treatment of Jurkat T-cells primarily affect the second wave of Vav phosphorylation (Geyeregger et al. 2005) that was shown to critically depend on lipid rafts (Valensin et al. 2002). Moreover, raft disruption by methyl-β-cyclodextrin treatment causes a pattern of disturbed immunological synapse formation similar to that obtained by PUFA treatment (Geyeregger et al. 2005).

3.3 The Other Side of T-cell-Mediated Immune Responses: PUFA Effects on APC Functions

In addition to the above discussed direct effects on T-cells, it has to be taken in account that PUFAs also affect APCs and thus T-cell mediated immune responses. Beyond blocking ativation of APCs such as dendritic cells (H Wang et al. 2007a; Weatherill et al. 2005; Zeyda et al. 2005b) or macrophages (JY Lee et al. 2003a) as assessed by Toll-like receptor induced signaling, surface molecule expression, and cytokine production, PUFAs also affect the antigen-presenting activity of APC, as reviewed in detail in (Shaikh and Edidin 2006).

PUFAs interfere with antigen-presentation on several levels. They have been shown to downregulate expression of MHC class I and II (Hughes and Pinder 1997; H Wang et al. 2007a; Weatherill et al. 2005), even though not in all studies (Erickson et al. 1997; Zeyda et al. 2005b). MHC molecules are concentrated in lipid rafts (Anderson et al. 2000) and pertubation of lipid rafts affects the antigen-presenting capacity of macrophages (Chakraborty et al. 2005). Hence it may be speculated that PUFA-mediated lipid raft alterations also affect antigen presentation. Indeed, PUFAs have recently been shown to lower B-lymphoblast susceptibility to lysis by alloreactive CD8+ T-cells, an effect depending on antigen presentation by MHC I (Shaikh and Edidin 2007) but a potential link to lipid raft alterations remains to be elucidated. In contrast to this study, PUFA-modified mouse tumor cells revealed increased sensitivity to cytotoxic T-cells in an earlier experiment (Jenski et al. 1993). These differences may be due to an overlay of different effects. For instance, PUFAs may differentially modulate MHC conformation and thus affinity to different

antigens (Jenski et al. 2000; Roof et al. 1990). Altogether, detailed mechanisms and effects of PUFA action on antigen presentation are largely unexplored yet.

4 Conclusions, Outlook

A plethora of studies exists on PUFA effects on inflammatory and autoimmune diseases and many of them have found effects on T-cell functions and signaling. However, many of the clinical studies are underpowered and effects often are modest. There is hence still a need for appropriately powered clinical studies evaluating PUFA effects including elucidation of the underlying molecular mechanisms. The discovery of mechanisms underlying the beneficial effects of PUFA in a variety of disorders could open novel strategies for direct and/or more efficient targeting by pharmaceutical compounds. For instance, the understanding of PUFAs on T-cell membrane organization is growing and a specific targeting of LAT localization in lipid rafts could be an approach to efficiently and selectively block T-cell activation. On the other hand, relatively little is known about membrane modulation of APCs including MHC conformation, spatial organization, and trafficking. Also PUFA effects on interleukin-induced signaling are not sufficiently clarified yet. Thus, research on mechanisms of PUFA action on T-cells is warranted yet.

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Omega-3 Polyunsaturated Fatty Acids and Immunosenescence

Christopher A. Jolly and Sirisha Karri

Contents

Abstract: It is well known that omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) are considered anti-inflammatory while omega-6 polyunsaturated fatty acids are proinflammatory. Research elucidating the mechanisms of ω-3 PUFA actions has focused largely on the T-cell. ω-3 PUFA have been shown to regulate the balance of T-cell subsets as well as the intracellular signaling pathways regulating T-cell proliferation. This results in the suppression of proinflammatory cytokines like interleukin-2 and interferon gamma in models of increased inflammation. ω-3 PUFA have also been shown to modulate T-cell function indirectly by influencing the ability of key antigen presenting cells like macrophages and dendritic cells to provide the necessary activating signals to T-cells and other immune cells. Interestingly, in the few models of immunosuppression that have been studied, ω-3 PUFA seems to increase immune function bringing the response to near normal levels. One key area where very few studies have been performed is the impact of ω-3 PUFA on the aging immune system. The important point to keep in mind in general is that regardless of the mechanisms, ω-3 PUFA feeding or supplementation has not been shown to have any clear deleterious effects in short-term (less than 6 months) studies.

Keywords: Omega-3 fatty acid • T-cell·inflammation • Rodent Diet

1 Introduction

Polyunsaturated fatty acids (PUFAs) can be divided into two major classes. First, omega-3 (ω-3) or n-3 PUFAs are well known for their anti-inflammatory properties (Calder, 2003). The other major class, the omega-6 PUFAs, are considered proin-

C. A. Jolly (✉) · S. Karri
Division of Nutritional Sciences
1 University Station – A2700
The University of Texas at Austin
Austin, TX 78712
Tel.: (512)471-7290
Fax: (512)471-5844
E-mail: jolly@mail.utexas.edu

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flammatory. The ω-3 and omega-6 PUFA differ structurally because of the position of their double bonds on the carbon chain. Omega-6 PUFA have double bonds starting six carbons from the methyl end of the carbon chain while ω-3 PUFA have double bonds starting three carbons from the methyl end. The two major omega-6 PUFAs that are typically consumed in the diet are linoleic acid (18:2; n-6; LA) and arachidonic acid (20:4; n-6; AA). Western diets are overwhelming composed of omega-6 PUFA with only small amounts of ω-3 PUFA being consumed. The three major ω-3 PUFAs are α–linolenic acid (18:3; n-3; α-LNA), eicosapentaenoic acid (20:5; n-3; EPA) and docosahexaenoic acid (22:6; n-3; DHA). It is important to note the alpha in front of linolenic acid (18:3; n-3) so as not to be confused with γ–linolenic acid which also is 18:3 but is an n-6 fatty acid. The PUFAs LA and α-LNA are considered essential fatty acids because they cannot be synthesized in mammals. Starting with LA humans can sequentially synthesize γ–LNA then AA. Starting with α–LNA humans can synthesize EPA then DHA. However, the major concern is whether or not humans can synthesize enough EPA and DHA from α–LNA.

Data clearly shows that when mammals are fed diets enriched in LA there is a dramatic increase in AA in tissues. This indicates that LA is fairly efficiently converted to AA in tissues, which is important since the LA rich corn oil is commonly used in Western diets. On the other hand, if mammals are fed diets enriched in α–LNA there is very little change in EPA or DHA which indicates that EPA and DHA must be consumed directly to significantly increase their tissue levels. An important point needs to be made here. Studies examining the conversion rate of LA or α–LNA to their subsequent longer chain metabolites are carried out in relatively short term studies lasting weeks to a few months. The question still remains as to whether or not long term (years) consumption of α-LNA from rich sources like flaxseed oil may lead to the gradual build up and maintenance of EPA and DHA in tissues. This has lead to the marketing of fish oil supplements derived from cold water marine fish, which are the richest direct source of EPA and DHA.

We and others have shown in rodent models that EPA and DHA are more potent in terms of their anti-inflammatory properties than α–LNA (Collison et al. 2005). In fact, it is suggested that DHA may be more potent than EPA as shown by studies in rodents comparing purified EPA and DHA feeding. This is why research on n-3 PUFAs now focuses more on the mechanisms of action of DHA. When comparing the impact of EPA and DHA feeding on inflammatory diseases in rodent and human studies it is quite clear that the n-3 PUFAs are more potent in the rodent studies. This is primarily due to the fact that EPA and DHA feeding are often started before or at the same time as the start of the inflammatory disease. In contrast, in human clinical trials the inflammatory disease is already in progress when n-3 PUFA supplementation begins. Even though n-3 PUFA supplementation does have beneficial anti-inflamatory effects in clinical trials, it is quite clear that to obtain the maximum beneficial effects of n-3 PUFAs it should be used as a prophylactic agent (Chapkin et al. 2000).

In the last few years, it has become clear that inflammation plays a pivotal role in many major diseases including heart disease, certain types of cancer and obesity. When thinking about which immune cell type to study to gain insight into the anti-inflamatory mechanisms of n-3 PUFAs, the first to come to mind is the T-cell.

Indeed, the T-cell is by far the most heavily studied immune cell when examining n-3 PUFA effects. This is because the T-cell can easily be isolated from rodents and human peripheral blood in significant numbers, is extensively studied for cell signaling and gene expression mechanisms and is pivotal in determining the type and extent of immune response. The macrophage comes in second with a few studies in other immune cells like the neutrophil and natural killer cells. The macrophage, similar to the T-cell can be isolated in significant amounts from human peripheral blood and rodents while it is difficult to get significant numbers of other immune cell types for extensive experimental analysis. Therefore, the bulk of reviews and discussions on the anti-inflammatory mechanisms of n-3 PUFAs center around the T-cell and to a lesser extent the macrophage. The mechanisms by which n-3 PUFAs impact T-cell function can be divided into two major categories: direct versus indirect effects. Direct effects include regulating events within the individual T-cell like membrane structure and signal transduction which are discussed in detail in other chapters and therefore we will only briefly discuss here. This may lead to shifts in the proportions of T-cell subsets which will have a dramatic impact on the type and magnitude of an inflammatory response. Indirect effects include regulating eicosanoid production and accessory cell function that can impact T-cell function.

2 Omega-3 PUFA Anti-inflammatory Effects in Disease States

The potent anti-inflammatory effects of dietary fish oil has lead to several studies in humans and rodent models examining the role of EPA and DHA to improve other disease states or situations in which a strong inflammatory response may be detrimental through regulating the immune response (Sijben and Calder, 2007). In fact, results have been promising enough that supplementation of total parenteral nutrition (TPN) formulas with ω-3 PUFAs may be beneficial in patients susceptible to deleterious hyperinflammatory responses like surgical or critically ill patients or those with sepsis (Calder, 2006). However, this may not prove to be true in all clinical situations of TPN as a recent study in rats showed that fish oil supplementation to TPN formulas administered to at the time of total gastrectomy and for 3 additional days afterwards actually enhanced macrophage phagocytic activity and T-cell IFN-γ production (Lin et al. 2006). This may be due to the impact of the altered physiological condition (type of procedure, disease type) on the immune system. For example, it was recently shown that T-lymphocyte proliferation was suppressed to the same extent by ω-3 PUFAs cultured in vitro with peripheral blood mononuclear cells from healthy and diabetic subjects. In contrast, the T-lymphocytes from diabetic patients exhibited only half the reduction of IL-2 production as seen in the healthy subjects (Alnajjar et al. 2006). Similarly, ω-3 PUFA feeding decreased T-cell IL-2 production and NF-κB activation in a small intestine rat transplant model (Wang et al. 2007b). Two cancer studies examining the impact of ω-3 PUFA supplementation in humans and rats also show promising results. First, ω-3 PUFA supplementation starting 5 days before surgery in patients undergoing colorectal

cancer surgery showed that the postoperative state exhibited a decreased Th-1/Th-2 T-cell ratio while ω-3 PUFA maintain the preoperative Th-1/Th-2 ratio (Matsuda et al. 2006). In a rat tumor transplant model, it was shown that feeding ω-3 PUFA for 8 weeks prior to injecting the cancer cells for tumor development showed decreased T-cell proliferation while macrophage function (hydrogen peroxide production) was increased. The association in this model with increased survival and decreased tumor burden suggests that enhance macrophage activation may play a key role in the anti-tumor effects of ω-3 PUFA (Pizato et al. 2006).

Interestingly, in a rat model of gestational type I diabetes found a differential effect of ω-3 PUFA feeding during pregnancy between the mothers and offspring. The mothers had elevated expression of IL-4 and IL-10 (Th-2 cytokines) while the offspring had elevated IL-2 and IFN-γ expression (Khan et al. 2006). Similarly, diabetes induced during pregnancy in rats showed that the obese offspring had reduced T-cell proliferation while ω-3 PUFA feeding to the mothers restored T-cell proliferation to normal levels (Guermouche et al. 2004). Similarly, in a rat sepsis model using colon ligation, it was shown that ω-3 PUFA feeding enhanced immune function as noted by increased IL-4 production and increased IgA levels suggesting the skewing of the immune response towards a Th-2 phenotype (Lin et al. 2007). An increased Th-2 phenotype (IL-10 levels), but T-cell proliferation was decreased, was also shown in neonatal piglets receiving DHA supplemented formula followed by influenza-virus immunization (Bassaganya-Riera et al. 2007). Several studies in aged mice show that ω-3 PUFA increase Th-1 cytokine production and inhibit Th-2 cytokine production (Watson et al. 2005). This is quite interesting because aged mouse Th-1 cytokine production is decreased while Th-2 cytokine production is increased. Not all studies show a significant effect of ω-3 PUFA supplementation feeding on the immune system. For example, in subjects following exercise, the beneficial effects of ω-3 PUFA feeding was not clear as it was recently shown to not have an effect following 12 weeks of walking three times per week and consuming DHA (Hill et al. 2007). In nonsurgical patients receiving enteral nutrition, adding ω-3 PUFAs slightly increased (nonsignificantly) natural killer cell activity and CD4/CD8 ratios (Sakurai et al. 2006). This suggests that unless there is a specific challenge or insult to stimulate the immune system, then the effects of ω-3 PUFA feeding are minimal. It is evident from these recent studies that the affects of ω-3 PUFA feeding can be quite variable depending on the physiologic condition being examined. However, the majority of the studies favor an anti-inflammatory effect and, most importantly, there has not been any evidence of a deleterious effect caused by the ω-3 PUFA. It is clear that in order to optimize the potential beneficial effects of ω-3 PUFAs, it is imperative to determine which immune cells are primarily impacted and how events within these cells are altered.

Another exciting area that is beginning to be examined in depth is the association between major depression and decreased ω-3 PUFA levels? Recently, it was found that decreased ω-3 PUFA and increased omega-6 PUFA levels in chronic fatigue syndrome was associated with decreased T-cell activation markers like the expression of the CD69 receptor. Even more interesting was the positive correlation between increased disease severity and increased ω-3 PUFA levels (Maes et al.

2005). This study reinforces that ω-3 PUFA effects can also involve indirect actions via reducing omega-6 PUFA tissue levels.

3 Omega-3 PUFA Affects Within the T-cell

The most difficult aspect of research on ω-3 PUFAs is elucidating the direct target of the immunomodulatory actions in key immune cells like T-cells. ω-3 PUFAs are well known to increase membrane fluidity due to their highly unsaturated carbon chains but this is a very general effect and cannot easily explain the differences observed physiologically between other PUFAs like AA and LA that also have unsaturated carbon chains(Chapkin et al. 2000). The discovery and isolation of lipid rafts has proven to be a very excited new area of research that gives a defined target within membranes that ω-3 PUFAs, especially DHA, may target? Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids and are thought to serve as the platform for cells, T-cells being a good example, to recruit and organize plasma membrane receptors and intracellular signaling components for cellular responses to external stimuli. Indeed several key immune receptors have been shown to aggregate into lipid rafts like T-cell receptors (TCR) and B-cell receptors (BCR). We will briefly discuss the impact of ω-3 PUFAs on lipid rafts, which have primarily been done in T-cells, because indepth discussions of fatty acid and lipid signaling effects on immune cells can be found in other chapters of this book.

Most of the work attempting to elucidate the biochemical and molecular mechanisms of how ω-3 PUFAs regulate inflammation have focused on the T-cell using diet studies in young healthy adult rodents or in some cases adding fatty acids in vitro to cell lines with the Jurkat human T-cell line being one of the most common cell culture models. The key events required for a proper T-cell response is stimulation of the T-cell antigen receptor (TCR) and CD28 costimulatory receptor. This leads to IL-2 production, which is a potent autocrine and paracrine growth factor driving T-cell proliferation. Thus T-cell proliferation (i.e. function) can be inhibited by altering TCR signaling and/or IL-2 signaling. The bulk of studies use polyclonal mitogens like anti-CD3 antibody to stimulate T-cells ex vivo or in vitro. Polyclonal mitogens will activate most all the T-cells in culture whether they belong to a subset of the CD4 or CD8 lineages. Some of the more recent studies are moving into models of antigenic stimulation which leads to the activation of a specific subset of T-cells but is better representative of an in vivo immune response. It is important to keep in mind that both types of stimulation protocols are important because the polyclonal activation provides a strong enough signal with enough T-cells responding in order to dissect the signaling mechanisms being regulated.

Indeed, ω-3 PUFAs added in vitro inhibit IL-2 receptor expression and subsequent signal transduction via the JAK-STAT pathways in both human cell lines (Li et al. 2005) and peripheral blood (Gorjao et al. 2007). The ω-3 PUFAs are thought to exert their inhibitory effects by changes membrane structure via incorporation into

individual phospholipid species (Li et al. 2005). This, in turn, may impact the ability of lipid rafts to form or impact the rafts' ability to recruit in or keep out key pro and antiproliferative signaling molecules. An important aspect of in vitro fatty acid work that is often debated is the fatty acid concentration used. As in these studies a 20–50 μM range is typical. However, doses up to 100–200 μM have also been reported in other studies. Indeed, fatty acid concentrations like this can be obtained at specific sites of inflammation but ω-3 PUFA blood content typically will increase to a maximum of about 10 μM following fish oil consumption (Chapkin et al. 2000). This is important to keep in mind to explain why the dramatic effects seen in vitro are often not as strong in vivo. It does raise the intriguing question of whether long term fish oil consumption could actually lead to the accumulation of ω-3 PUFAs in tissues such that the magnitude of the in vivo effects would be more similar to the in vitro effects. This does not mean that the in vitro studies lack value, in fact they are highly valuable for elucidating mechanisms and thus higher fatty acid concentrations are used in order to clearly identify alterations in membrane structure and signal transduction. The in vitro studies are also important to help gain insight into what fatty acid ranges are tolerated by T-cells and what cellular ω-3 PUFA levels are the most beneficial.

Some of the best studies to date for elucidating the mechanism(s) by which ω-3 PUFA feeding modulate T-cell function were conducted by Chapkin's group in young healthy mice. They have shown that feeding highly purified DHA can suppress splenic T-cell diacylglycerol and ceramide production and subsequent proliferation? They have followed this up with studies determining which T-cell subset is primarily impacted. The data in fish oil fed mice clearly show that Th-1 polarization in vitro is suppressed while having very little impact on Th-2 polarization (Zhang et al. 2005). The previous study used polyclonal stimuli (cytokines to skew T-cell polarization) to induce proliferation of T-cell subsets and recently have confirmed the inhibition of Th-1 subset proliferation in vitro and in vivo models of antigen specific stimulation (Zhang et al. 2006). In IL-10 knockout mice, feeding fish oil led to enhanced IFN-γ production while IFN-γ production was suppressed in wild type mice (Ly et al. 2005). This data suggests that part of the down-regulation of ω-3 PUFA on the Th-1 phenotype is due indicectly to enhanced Th-2 phenotype which can then suppress Th-1 function. In another group of studies in a mouse model that show ω-3 PUFA reduce the ability of the host to fight *Listeria Monocytogenes* infection they found that effector and memory T-cells were not altered and could still induce resistance following adoptive transfer (Irons et al. 2005). Similarly, antigen specific CD8 T-cell proliferation was not impacted (Irons and Fritsche, 2006). The lack of effect on T-cell function is quite surprising since there was a dramatic decrease in resistance to infection. One explanation may be that Chapkin's group used C57BL/6 mice while the infection studies were performed in BALB/c mice. The C57BL/6 mice, immunologically, have a strong Th-1 response while BALB/c mice are the opposite having a strong Th-2 response and weaker Th-1 response. Therefore, the differences in the effects of ω-3 PUFA may be due to the immunologic background of the mice. Alternatively, the differences could be related to amount of ω-3 PUFA in the diet. The Chapkin group fed 5% ω-3 PUFA by weight while the other studies used 50% ω-3 PUFA by weight. Therefore, the infection

studies may show a negative effect of ω-3 PUFA feeding because it is an extremely high fat diet.

The mechanism by which ω-3 PUFA, especially DHA, regulates T-cell subset function directly is thought to involve disruption of proper lipid raft formation. It has recently been shown that DHA feeding to young healthy mice can significantly alter the fatty acid content of lipid rafts and most notably a dramatic decrease in sphingomyelin content (Fan et al. 2004). This DHA induced change in lipid raft composition was associated with decreased recruitment of PKC-θ to the lipid raft and decreased AP-1 and NF-κB activation (Fan et al. 2004). Similar results adding DHA and EPA in vitro have shown decreased the translocation of PKC-α and –ε to the plasma membrane which is followed by decreased NF-κB activation in Jurkat T-cells (Denys et al. 2005). The translocation of PKC from the cytoplasm to the plasma membrane is used as a marker of activation since PKCs must interact with membrane lipids to be activated. These findings are very significant because PKC-θ is considered to be the key PKC isoform for T-cell activation and NF-κB is thought to be the key proinflammatory transcription factor. The importance of the lipid raft may be its role in the proper formation of the immune synapse which is the sight of contact between the T-cell and the antigen presenting cell. Indeed ω-3 PUFA have been shown to inhibit appropriate immune synapse formation in vitro (Geyeregger et al. 2005) which may be due in part to alterations in the organization of key cytoskeletal components like L-selectin at the immune synapse (Leid and Jutila, 2004).

One line of studies as examined an intriguing question in that could the anti-inflammatory properties of ω-3 PUFAs be enhanced combining them with other dietary regimens. For example, calorie restriction, similar to fish oil feeding, has potent anti-inflammatory effects. Calorie restriction is defined as a 40% reduction in food intake while maintaining proper vitamin and mineral intake and is also a potent antiaging strategy (Jolly, 2004). There have been several studies in autoimmune prone mice showing that a combination of calorie restriction and fish oil supplementation can have additive benefits (Jolly, 2005). The mice used in these studies develop severe autoimmune kidney disease and succumb to this disease at approximately 10 months of age. For example, fish oil plus calorie restriction was more effective than either dietary regimen alone at delaying the onset of autoimmune disease and therefore increased lifespan (Jolly et al. 2001a). This was associated with dramatic impacts on the immune system, especially the T-cell, because the dramatic rise in activation induced apoptosis and proinflammatory cytokine production caused by disease were all blunted (Jolly et al. 2001b). Similarly, the disease induced increase in proinflammatory cytokines and NF-κB activation in the kidneys was also reduced (Jolly et al. 2001a). The mice were put on the diets prior to developing autoimmune disease which highlights that ω-3 PUFA supplementation is the most beneficial when started prior to disease onset.

The effects described above were observed in studies using either fish oil (containing both EPA and DHA) or DHA alone. It is correct that, as mentioned previously, DHA is considered to be more potent than EPA however there have been several studies in which EPA was also shown to have potent anti-inflammatory effects. It

is worth talking about EPA because the possibility exists that perhaps EPA's effects on the immune system are different or unique from DHA. For example, both DHA and EPA (fed separately) were shown to suppress murine T-cell proliferation to a similar extent but EPA and not DHA could decrease CTLA-4 expression (Ly et al. 2006). CTLA-4 is an important adhesion receptor on T-cells and thus EPA may help to increase the potency of DHA's effects. This is supported by evidence in Jurkat T-cells supplemented in vitro with either EPA or DHA. Gene microarray analysis of these cells showed that DHA and EPA have both unique effects and some overlapping effects on the expression of a wide array of genes important in inflammation (Verlengia et al. 2004). However, the limited effects of EPA on basic responses like T-cell proliferation in humans (Miles et al. 2004; Miles et al. 2006) and T-cell antigen specific responses in mice (Barber et al. 2005) have kept EPA from being as intensely investigated as DHA. Since EPA has been shown to have some benefits in certain disease conditions like tumor growth (Kimura and Sumiyoshi, 2005), it is possible that EPA may have unique, as yet unidentified, effects in select disease conditions where the effect is more on nonimmune cells.

4 Omega-3 PUFA Affects on Accessory Cells

Omega-3 PUFAs have also been shown to indirectly inhibit T-cell function by altering the ability of the T-cell to be properly stimulated by accessory cells. The two best known accessory (antigen presenting) cells (APC) are the macrophage and the dendritic cell. PUFAs have been shown to alter MHC I and II expression on APCs and the expression of TCR and T-cell expressed adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and leukocyte function associated antigen–1 (LFA-1; Shaikh and Edidin, 2006). The altered expression can be due to either decreased plasma membrane expression of the receptor or the inappropriate assembly of the receptors at points of T-cell:APC contact. All these receptors are important in forming the interaction between the APC and T-cell for an appropriate immune response.

A key new mechanism that has been shown to explain ω-3 PUFA inhibition of T-cell function is the inhibition of toll like receptors (TLRs) found on dendritic cells. TLRs are a group of receptors that recognize lipopolysaccharides (LPS) and are, therefore, important for activating naïve T-cells in response to microbial infections. In vitro evidence shows that saturated fatty acids increase dendritic cell function while DHA inhibits dendritic cell function. For example, saturated fatty acids added in vitro increase the expression of key costimulatory receptors on dendritic cells like CD86 and CD40 and increase the production of the important proinflammatory cytokines IL-12 and IL-6, which is inhibited by DHA (Weatherill et al. 2005). These results were corroborated in vitro by showing that both EPA and DHA also had inhibitory effects on human dendritic cell function (IL-12 and IFN-γ production; Wang et al. 2007a). This is especially important because the dendritic cell is considered to be an important cell in bridging the innate and adaptive immune response via

converting a general recognition of microbial cell wall components into a specific T-cell response (Barton and Medzhitov, 2002).

It is important that when thinking about accessory cell impacts on immune function, we also think about the contribution of nonimmune cell types. It is now clear that cytokines and other immunomodulatory molecules can be produced by nonimmune cells/tissues and since ω-3 PUFAs consumed in the diet can be incorporated into all tissues (albeit to varying degrees) there can be more indirect effects on immune function in vivo. For example, cytokine (IL-8, RANTES) and PGE_2 production by respiratory airway cells is decreased by DHA feeding which could have a dramatic anti-inflammatory effect on airway diseases like allergy (Bryan et al. 2006). The role of eicosanoids is discussed in more detail below.

5 Omega-3 PUFA and the New Eicosanoids

The first known mechanism elucidated in the 1970s to explain, in part, the anti-inflamatory properties of the n-3 PUFAs EPA and DHA were by decreasing the production of prostaglandin E_2 (PGE_2) and leukotriene B_4 (LtB_4). There are two major mechanisms by which EPA and DHA can decrease PGE_2 and LtB_4 production. First, both EPA and DHA will compete with AA in membranes thus decreasing the amount of the parent fatty acid (AA) for eicosanoid production in membrane phospholipids. Second, EPA but not DHA can lead to less biologically potent forms of PGE_2 and LtB_4 called PGE_3 and LtB_5, respectively. This is significant because both PGE_2 and LtB_4 are potent pro-inflammatory eicosanoids helping to recruit immune cells like the T-cell to sites of inflammation. There is one interesting caveat with PGE_2 in that the pro-inflammatory properties are attributed to effects on nonT-cell cells like increasing vascular permeability, however, PGE_2 will directly inhibit T-cell proliferation if allowed to directly bind to T-cell PGE_2 receptors. Thus, changes in PGE_2 production by tissues can explain decreased inflammation in vivo but cannot explain reduced T-cell proliferation directly which is typically seen ex vivo following EPA and DHA feeding/supplementation (Chapkin et al. 2000). Therefore, both PGE_2 and LtB_4 are important at initiating and enhancing inflammation and recent new exciting evidence suggests that n-3 PUFAs can also inhibit inflammation/T-cell function by producing eicosanoid metabolites that will turn off the inflammation by inhibiting T-cell function (i.e. promote resolution of the inflammatory response; Ariel and Serhan, 2007).

Eicosanoids, by definition, are derived from twenty carbon fatty acids like AA and EPA but not DHA since it is 22 carbons long. Recently, it has been discovered that DHA can also be converted to a docosanoid (metabolite of 22 carbon fatty acids) via the lipooxygenase pathway. This docosatriene is termed protectin D1 (PD1) and has been shown to be produced under conditions in which peripheral blood mononuclear cells were skewed towards a Th-2 phenotype. PD1 was shown to inhibit T-cell migration and TNF-α and IFN-γ secretion (Ariel et al. 2005). Work in both humans and mice, supports the anti-inflammatory functions of PD1. A recent

study showed that human asthmatics had low PD1 levels in lung exudates when the disease was exacerbated and that PD1 administration inhibited T-cell recruitment and proinflammatory cytokine production was decreased in a mouse model of airway inflammation (Levy et al. 2007).

6 Conclusions

In general, ω-3 PUFAs are considered anti-inflammatory while omega-6 PUFAs are considered proinflammatory. This leads one to assume that ω-3 PUFAs are strictly associated with suppressing immune cell function. It is quite clear that there are many examples in healthy young rodents and disease models that ω-3 PUFA inhibit T-cell, macrophage and dendritic cell function. However, under certain circumstances like exercise or in cases where the immune system is suppressed you may see no effect or an actual increase in immune cell function. This gives promise for ω-3 PUFA supplementation in the elderly however there are few studies to indicate what impact, if any, ω-3 PUFA supplementation will have in this population. The varying effects of ω-3 PUFA seems somewhat confusing which may due to the fact that we do not completely understand the cellular and molecular mechanisms by which ω-3 PUFAs exert their effects. The regulatory role of ω-3 PUFAs is complex in vivo in that there are effects on immune cells (T-cells, macrophages, dendritic cells) and nonimmune cells in tissues in and around the site of infection or insult that together exert their anti-inflammatory properties (Calder, 2007). The potential importance of ω-3 PUFA supplementation has reached a higher level since it is now clear that inflammation and inflammatory cytokines may play a pivotal role in many major diseases like heart disease, obesity and certain types of cancer. In order to maximize the use of ω-3 PUFAs in disease treatment it is imperative that the mechanisms by which inflammation is regulated be understood in order to determine the disease conditions that can be improved and optimize the dose or amount of ω-3 PUFAs needed for optimum health benefits. However, regardless of the mechanism or whether a specific cell type is being inhibited or stimulated, ω-3 PUFA will typically correct the dysfunction or in the very least have no effect at all.

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Effect of Intrinsic and Extrinsic Lipids on T-cell Signalling

Anis Larbi, Emilie Combet, Graham Pawelec and Tamas Fulop

Contents

Abstract: T-cell activation is dependent on activating and inhibitory signals and cell fate is influenced by the interplay between different these different signalling pathways. Because proximal events are relayed through the membrane via specific membrane microdomains called rafts, the lipid composition of the plasma membrane critically influences signal transduction and thus cellular functions. Rafts are highly motile domains, enriched in cholesterol compared to the rest of the membrane. Their specific lipid composition makes these domains very sensitive to external changes such as variations in the cholesterol, saturated and unsaturated fatty acid content of the immediate environment. Immune cells, and in particular T-cells, depend on membrane raft integrity for initiating signalling, so dysregulation of the processes involved in the maintenance of an adequate lipid environment is likely is to be a significant modulator of immune functions. In this chapter, we will review the modulation of

A. Larbi (✉) · G. Pawelec
Center for Medical Research (ZMF)
University of Tübingen Medical School
Waldhörnlest. 22 D-72072 Tübingen
Germany
Tel: +49 (0)7071 2981269
Fax: +49 (0)7071 294677
E-mail: anis.larbi@medizin.uni-tuebingen.de

E. Combet
Section of Medicine and Therapeutics
Western Infirmary, University of Glasgow
Scotland

T. Fulop
Research Center on Aging
University of Sherbrooke, Qc
Canada

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TcR-dependent signalling events by lipids in *in vitro* and *in vivo* models focusing on the involvement of membrane rafts. Clinical cases such as autoimmune diseases, aging and Alzheimer's disease will be used to illustrate recent findings in this field.

Keywords: T-cell Signalling • Membrane rafts • Cholesterol • Lipoproteins • T-cell dysfunction • Immune senescence

1 T-cell Signalling

T-cell activation is dependent on complex signalling events: some signalling pathways act independently while others cross-talk to form a very intricate signalling map. Signal transduction starts at the plasma membrane, where very discrete domains enriched in cholesterol, the membrane rafts, drive the first steps of signalling molecule interaction and activation (He et al. 2005; Harder 2004). The

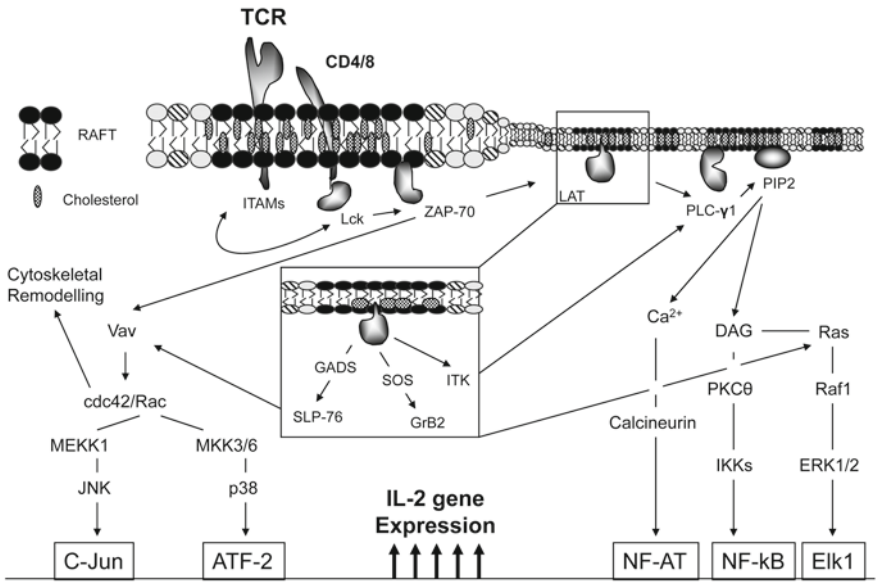

Fig. 1 T cell receptor signalling
T cell receptor ligation induces the phosphorylation of immuno-tyrosine based activation motifs (ITAMs) which are recognized by the CD45-activated protein tyrosine kinase, Lck which in turn activates the Zeta associated protein of 70 kDa (ZAP-70). The recruitment of the Syk family member, ZAP-70 induces the phosphorylation of the Linker of Activated T cells (LAT). LAT has no intrinsic activity but is an adaptor protein associated to many other molecules. This is the scaffolding role of LAT. The resulting signalling is inducing the translocation of transcription factors to the nucleus which allows the regulation of IL-2 gene. T cell activation is then achieved. GADS is an adaptor protein while SOS is a guanine exchange factor working with GrB2. ITK: inducible tyrosine kinase, DAG: diacylglycerol, PKC: protein kinase C, PIP2: phosphatidylinositol-4,5-bisphosphate, SLP-76: SH2 domain containing leukocyte phosphoprotein of 76 kDa, IKK: inhibitor of kappa B factor kinase, ERK: extracellular signal–regulated kinase.

subsequent formation of the immune synapse is required for sustained signalling resulting in activation (Dustin et al. 2005). The phosphorylation of cytoplasmic signalling molecules induced by membrane raft activation is regulated by many protein tyrosine kinases, which ultimately leads to the translocation of transcription factors to the nucleus, where they influence gene expression (Cannons et al. 2004). All these events of T-cell receptor (TcR) signalling are detailed in Fig 1.

2 Membrane Rafts, Lipids, and T-cell Signalling

The mammalian cell membrane consists of a lipid bilayer, composed primarily of phospholipids and cholesterol (Spector 1985). The main phospholipids present in the mammalian cell are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) (31%, 29%, 13%, 26% respectively), accompanied by low amounts of phosphatidylinositol (PI) (Yawata et al. 1984). Sphingomyelin, the only phospholipid not derived from glycerol, is composed of a sphingosine, a fatty acid, a phosphate group and a choline. It is located, along with PC in the exoplasmic (outer) leaflet, while PE, PS and PI are located to the cytoplasmic (inner) leaflet, a distribution at the heart of the compositional asymmetry of the plasma membrane. While phospholipids can display a rapid lateral diffusion across a given layer, their movement from layer to layer is limited and enabled only by the enzymes phospholipid flippase/floppase/scramblase (Dolis et al. 1997; Bevers et al. 1999; Daleke and Lyles 2000; Sims and Wiedmer 2001). Cholesterol fits between the gaps created by the sphingomyelin molecules, resulting in membrane regions rich in sphingomyelin and cholesterol, which can be termed lipid rafts, the formation of which is the result of different affinity between lipid molecules, or lipid and membrane proteins (Wassall et al. 2004). Several extracellular proteins with either a GPI anchor or a myristoylated or palmitoylated tail are associated with lipid rafts, which have an important role in signal transduction.

Since the first selective isolation of lipids by treatment with Triton X-100 in 1974 many experimental protocols have been adjusted to the different cellular models studied (Kirkpatrick et al. 1974). The floating fraction resulting form gradient centrifugation is easily harvested and is highly enriched in lipid-anchored proteins such as glycosylphosphatidylinositol-anchored proteins (located at the outer face of the plasma membrane) and proteins with post-translational modifications such as acylation or palmitoylation (located at the inner face of the plasma membrane) (Blank et al. 2002). Sphyngomyelin, cholesterol, saturated phospholipids, glycolipids, glycerophospholipids (choline, ethalonamine, serine, phosphatidylserine, phosphatidylcholine), diacylglycerol, palmitic acid and stearic acid are preferentially targeted to membrane rafts (Rouquette-Jazdanian et al. 2002). The maintenance and functionality of membrane rafts is dependent on the sphyngomyelin:cholesterol ratio, explaining why cholesterol extraction, which will be described later, may influence cellular signalling and processes (Helms et al. 2004).

Cellular cholesterol metabolism is tightly regulated and although no clear mechanisms have been demonstrated in T-cells, studies suggested that cholesterol influx, efflux, synthesis, use and degradation are the same in all mammalian cells (Fielding et al. 2001). Most of the cholesterol (up to 95%) is targeted to the membrane; T-cell proliferation triggers a need for cholesterol, explaining the increase in the cholesterol synthesis rate at that time. The membrane raft marker, ganglioside M1 (GM1) was also shown to be highly synthesized following proliferation induced by TcR triggering (Tuosto et al. 2001). Thus, cholesterol and ganglioside synthesis rates are correlated and associated with cell cycle progression induced by positive signalling.

Lipid metabolism is highly relevant to cell cycle regulation and these downstream events are strongly influenced by upstream events (signalling) which are easily influenced by the lipid environment (Eyster 2007). Glycerophospholipids, major structural components of the cell membrane, "contain 2 fatty acid" chains attached to a glycerol backbone; the unsaturated fatty acids are usually incorporated in position sn-2, while saturated fatty acids are incorporated in position sn-1 (Anderson and Sperling 1971). The length and saturation level of the fatty acid acyl chain affects the biophysical properties of the membrane bilayer, including membrane stability, permeability, fluidity and curvature. The occurrence of n-6 polyunsaturated fatty acids in the phospholipids of human mononuclear cells is approximately 6–10% linoleic acid (18:2, n-6), 1–2% di-homo-1,2,gamma-linolenic acid (DHGLA 20:3, n-6) and 15–25% arachidonic acid (20:4, n-6). n-3 polyunsaturated fatty acids are relatively rare, with traces of α-linolenic acid (ALA, 18:3, n-3), 0.1–0.8% of eicosapentaenoic acid (EPA, 20:5, n-3) and 2–4% of docosahexaenoic acid (DHA, 22:6, n-3) (Yawata et al. 1984; Calder 1998; Yaqoob et al. 2000; Calder 2001; Thies et al. 2001).

The fatty acid composition of the lipid membrane can be readily modified through dietary supplementation with fatty acids. Supplementation with fish oils resulted in an increased incorporation of DHA and EPA into the phospholipids of inflammatory cells, neutrophils, monocytes and lymphocytes, at the expense of arachidonic acid (Gibney and Hunter 1993; Marangoni et al. 1993; Yaqoob et al. 2000; Thies et al. 2001; Kew et al. 2003). The composition of lipid rafts and their environment was also modified following EPA supplementation, potentially affecting their function in signal transduction through displacement of acylated signalling proteins from the membrane lipid rafts, with EPA incorporated in both cytoplasmic (PS, PE, PI) and exoplasmic leaflets (SM, PC) (Stulnig et al. 2001; Li et al. 2006).

Twenty-carbon polyunsaturated fatty acids act as substrates for the synthesis of eicosanoids, a group of bioactive mediators including prostanglandins, prostacyclins, thromboxanes and leukotrienes (Calder 2001). Due to its predominance in the cell membrane phospholipids, arachidonic acid is the main substrate for eicosanoic production. Mobilisation of free arachidonic acid is achieved through the action of phospholipases on the membrane phospholipids. Phospholipase A2, the rate limiting step in the production of proinflammatory lipid mediators, cleaves phospholipids at the sn-2 position, liberating the fatty acid molecule, which can then act as a substrate for the synthesis of the eicosanoid mediators (Glaser et al. 1993; Spiteller 2002). The arachidonic acid produced is then metabolized via three major

pathways (Moncada et al. 1986; Belton et al. 2003) The enzyme cyclooxygenase (COX) is responsible for prostaglandin synthesis, with the COX-1 (constitutive) isoform responsible for basal synthesis, and the COX-2 isoform responsible for induced prostaglandin synthesis. The pathway leads to the formation of thromboxane A2 and the prostaglandin (PG) family (PGD2, PGE2, PGF2, PGI2). The enzyme lipoxygenase (LOX), including the 5, 12, and 15-lipoxygenases, converts the twenty-carbon polyunsaturated fatty acid to a labile hydroperoxy intermediate, leading to the formation of leukotriene (LT) A4-derived compounds such as LTC4, LTD4. LTE4, LTB4 and lipoxins. The final pathway involves the less-documented cytochrome P450 epoxygenase, and leads to the formation of epoxyeicosatrienoic acids (EETs), along with conjugated dienol and alcohol derivatives (Calder 2001; Zeldin 2001; Fleming, 2007).

All these lipid mediators derived from membrane phospholipids may lead to a broad range of effects on T-cells and surrounding cells. Following the inflammatory response, the level of prostanoid is increased and influences T-cell responses (Tilley et al. 2001). PGD2 was shown to be a chemoattractant, since T-cells, especially of Th2-type, express its receptor, CRTH2 (Nagata et al. 2003). Moreover, T-cells express the PGE2 receptors, EP1-4, which suggests that the broad receptor repertoire expressed by immune cells differently influences cell fate depending on the prostanoids present in the local environment (Tilley et al. 2001). PGD2 and PGE2 (via EP2 and EP4) induce the elevation of cAMP, which is associated with inhibition of effector cell functions while PDF2 and PGE2 (via EP1) induce calcium mobilization and T-cell activation (Sugimoto et al. 2007). The effect of prostanoids produced during an inflammatory response is determined by the array of receptors expressed and the intracellular pathways to which they are coupled. Further studies are needed to increase our knowledge of prostanoid signalling, cross-talk and balance, which provides either activating or inhibitory signals to T-cells. This has practical consequences; asthma is an example of the role of prostanoids controlling pro- and anti-inflammatory signals to T-cells in a clinically important context (Kostenis et al. 2006).

Modulation of inflammation can be achieved through n-3 Fatty acid supplementation: EPA inhibits the release of arachidonic acid by phospholipase A2, as well as its oxidation by COX, resulting in a reduced ability to synthesize arachidonic-derived eicosanoids (Obata et al. 1999). Fish oil supplementation resulted in a 50–60% decrease in PGE2 synthesis by mononuclear cells (Meydani et al. 1993; Caughey et al. 1996). Moreover, EPA-derived eicosanoids are considered less potent than those synthesised from arachidonic acid. n-3 Fatty acids, such as EPA and DHA, may alter T-cell functions through inhibition of the expression of cell surface molecules required for antigen presentation (Fujikawa et al. 1992; Hughes and Pinder 2000). Recent in vivo studies confirmed previous in vitro experiments showing that changes in serum lipid composition influence cell behaviour (Stulnig et al. 2004). Several groups reported the suppressive effects of polyunsaturated fatty acids (PUFAs) on T-cell proliferation, and we tested the hypothesis that this effect is the result of altered T-cell membrane properties and impaired TcR signalling (Larbi et al. 2005). The functionality of peripheral

T-cells before and 2 h after an intravenous infusion of heparin plus a polyunsaturated fatty acid (PUFA)-rich lipid emulsion were tested. We demonstrated a reduced peripheral T-cell membrane fluidity and altered lipid raft organization, both of which were associated with reduced T-cell proliferation after stimulation via CD3/CD28. Tyrosine phosphorylation of linker of activated T-cells (LAT) and activation of Akt in T-cells was also impaired (Larbi et al. 2005). Acute PUFA elevation was associated with a reduction in T-cell membrane cholesterol exchange with the cellular milieu ex vivo (Larbi et al. 2005). More information concerning PUFA and other fatty acids on T-cell function and signalling is given in detail by Stulnig et al. in this book.

3 Lipoproteins and Cholesterol Transport

A physiological process involved in the elimination of excess oxidized lipids is high-density lipoprotein (HDL) transport. HDL is highly abundant in the blood (between 1 and 1.5 mM) and functions as a cholesterol transporter from the tissue to the liver. HDL is composed of phospholipids and apolipoprotein A-1 (apoA-1) (Davidson et al. 2007). A kinetic model suggests that cholesterol efflux via apoA-1 is a two-step process (Gaus et al. 2001). In the first step, some of the plasma membrane cholesterol contributes to a fast initial efflux (over the first hour) and leads to a progressive and slow efflux pool over several hours. The rapid and slow cholesterol efflux pools represent cholesterol derived from raft and nonraft domains of the membrane, respectively, and are dependent on the association between ApoA-1 and the ATP-binding cassette A1 (ABCA1). This model is derived from experiments on macrophages, but no data are available on T-cells. These studies demonstrate the association of ApoA-1 to raft and nonraft domains of the macrophage plasma membrane. Cholesterol depletion, induced by 7-ketocholesterol and treatment with cyclodextrins, blocks apoA-1 binding to membrane rafts and inhibits cholesterol efflux from the slow pool (Gaus et al. 2004). This model is very attractive but must be critically demonstrated since apoA-1 does not exist in a free state in the periphery but is always associated with HDL, which may influence its binding to the discrete membrane rafts. This physiological approach is nonetheless very promising (Singh et al. 2007).

The physiological process of cholesterol transport via HDL and its associated protein apoA-1 is of major importance when considering the role of cholesterol in the maintenance of membrane integrity and fluidity. Any change in the biochemical properties of the plasma membrane will influence cell fate. To explore reduced T-cell function in aged individuals, we analyzed membrane raft properties and were able to demonstrate several deficiencies (Fulop et al. 2005; Larbi et al. 2004a; Larbi et al. 2006). The first data set suggests that changing the plasma membrane cholesterol content can influence the T-cell proliferative response to stimulation (Douziech et al. 2002). Reducing cholesterol content of T-cells from elderly individuals using methyl-β-cyclodextrin (an accepted cholesterol extractor) provided a partial

restoration of T-cell proliferative capacity. However, more detailed investigations failed to support the use of cyclodextrin to restore T-cell function, despite a restoration of membrane fluidity, because several side effects such as altered raft properties, and changes in signalling molecule association with rafts, where found (Larbi et al. 2004b). Other studies also suggested that cholesterol enrichment in T-cells had a suppressive effect on basic functions such as calcium mobilization, chemotaxis and proliferation (Nguyen et al. 2004).

4 Cholesterol-lowering Drugs and Immunity

While T-cell function was not restored in experimental models when the cholesterol level was restored, clinical studies have demonstrated an effect of cholesterol-lowering drugs (statins) on immunity. The statin drug family is used to prevent cardio-vascular diseases through inhibition of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis. Atorvastatin, Fluvastatin, Lovastatin and Simvastatin also known as Lipitor®, Lescol®, Mevacor® (the first to be marketed), and Zocor®, respectively are the best known and most often used statins (Shepherd et al. 2003). Statins prevent cardio-vascular diseases by improving endothelial function, modulating inflammatory responses, maintaining plaque stability and preventing thrombus formation (Furberg 1999). Some favorable effects of statins were observed in demented (Wolozin et al. 2007) and cancer patients (Khurana et al. 2007).

4.1 Neutrophils

Immune modulation by statins has also been recently reported. Atorvastatin was shown to reduce the risk of ischemia/reperfusion injury after renal transplantation (Gottmann et al. 2007). The improvement of endothelial function by increased nitric oxide synthase activity induced by statins is accompanied by a reduction in adhesion molecule expression, which might explain the reduced ischemia/reperfusion injury in a rat model (Cowled et al. 2007). Moreover, statin therapy prior to cardiopulmonary bypass reduced the level of circulating markers of inflammation and increased neutrophil apoptosis (Chello et al. 2007). In humans, clinical studies demonstrated the inhibitory effect of statins on neutrophil functions. A 6-week intake of Atorvastatin significantly reduced superoxide anion generation by resting and stimulated neutrophils (Kowalski et al. 2006). A longitudinal study also confirmed the above data, showing reduced IL-8 production and chemotactic activity following statin therapy (Guasti et al. 2006). However, there is still no clear evidence supporting the mechanism of action of statin in neutrophils.

4.2 T-cells

Another effect of statins is to block the production of isoprenoids required for post-translational modification of signalling molecules involved in immune cell activation. Globally, immune system activities are suppressed by statins which are thus considered as anti-inflammatory drugs interfering with the activation of proinflammatory cells such as macrophages and endothelial cells (Ghittoni et al. 2007). It is worth noting that during activation, the activity of HMG-CoA reductase is also enhanced, probably via protein kinase C (Chakrabarti et al. 1991). The role of HMG-CoA reductase and mevalonate production in T-cell activation and metabolism was shown with the use of Lovastatin which inhibited anti-CD3-induced T-cell mitogenesis in a dose-dependent manner (Chakrabarti et al. 1991). Interestingly, Lovastatin had no effect on T-cell proliferation in the first 12 h of culture, suggesting that mevalonate is required from mid-G1 into the late G1 phase of the cell cycle. Early and late events of TcR signalling including intracellular calcium mobilization, inositol phosphate production, and tyrosine phosphorylation of phospholipase Cgamma1, where shown to be inhibited by Lovastatin (Goldman et al. 1996). Moreover, post-translational processing of ras was disrupted, which influenced the ras-signalling pathway involving mitogen-associated protein kinase (MAPK).

The statin-induced prenylation of signalling molecules is one major factor influencing T-cell activation and function. The Ras- and Rho-dependent signalling pathways including extracellular signal-regulated kinases (ERK) and p38 activation, respectively, are both diminished by statins (Greenwood et al. 2003). A recent article suggested statin-induced T-cell anergy mediated by early and sustained phosphorylation of ERK1 involving the accumulation of the negative regulator p27 (Kip1) (Waiczies et al. 2005) The first study to describe changes in post-translational modifications of protein due to statins and membrane rafts was described by Gubina et al., showing the link between CD43 exclusion from membrane rafts and Lovastatin treatment of CD4+ T-cells (Gubina et al. 2002). Although the raft markers GM1 and GM3 where not altered in localisation at the uropod and leading edge, respectively, CD43 was not associated with membrane rafts (Gomez-Mouton et al. 2001).

The clinical applications of statin use have to be reconsidered following the recent discovery of their broad effects. Because of their anti-inflammatory properties, statins can be useful in patients with cardio-vascular risks (Casserly et al. 2004). This is the case also with Alzheimer patients who often suffer cardio-vascular problems (Crisby et al. 2002). Moreover, several autoimmune diseases were shown to be controllable by statin therapy. This is the case for chronic and relapsing experimental autoimmune encephalomyelitis and is now being tested in multiple sclerosis clinical trials (Weber et al. 2006). This anti-inflammatory effect was associated with reduced migration of leukocytes into the central nervous system, inhibition of MHC Class II expression and blockade of costimulatory signals required for activation of proinflammatory T-cells, induction of a Th2 phenotype in T-cells, and reduction in the expression of inflammatory mediators in the central nervous system (Stuve et al. 2003). Patients with systemic lupus erythematosus (SLE) also display dysregulation of T-cell signalling,

including the reduced expression of the protein tyrosine kinase Lck at the cellular level but its increased basal activation in membrane rafts (Jury et al. 2004). Atorvastatin treatment resulted in the reduction of the active form of Lck in resting T-cells and in the inhibition of Lck recruitment to the immunological synapse (Jury et al. 2006). Moreover, the production of IL-10 and IL-6 by T-cells (involved in SLE pathogenesis) was reduced when Atorvastatin was used. One peculiar characteristic of statins is their binding to an allosteric site on the LFA-1 alpha chain (Schramm et al. 2007). LFA-1 is involved in T-cell migration via the activation of the cytoskeletal machinery and activation of two transcription factors, c-Jun and c-Fos induced by MKK4/7 and MKK 3/6, respectively. Together, these data strongly suggest an immuno-modulatory role for statins in vitro and in vivo and future longitudinal studies will provide more accurate information on their suppressive effects in clinical use.

5 Lipids as Antigens

T-cell signalling complexity and specificity make it very sensitive to extrinsic factors. The first events of T-cell receptor (TcR) signalling rely on cholesterol-enriched membrane rafts and thus the lipid environment will have a major influence on this process. Lipids can enter passively through the membrane or can be actively bound by transporters such as lipoproteins. Therefore, the expression and function of receptors has an important role in the regulation of intracellular lipid composition. However, there is limited information on lipid-specific receptors in T-cells. Only the scavenger receptor CD36 (Lubick et al. 2006), which is expressed on most leukocytes, is known to facilitate the uptake of lipids either in their active form or after some modifications such as oxidation. In this respect, the accumulation of oxidized low density lipoproteins (oxLDL) may influence T-cell activation. Incubation of monocytes with oxLDL induced autologous T-cell proliferation while direct contact between T-cells and oxLDL induced their apoptosis (Fortun et al. 2001). Professional antigen presenting cells, dendritic cells (DC), were also recently shown to synthesize lipid antigens in response to bacterial stimulation and induce CD1-restricted T-cell activation through antigenic mimicry (Thurnher 2007). Thus, lipids derived from antigens such as viruses may have a potent immunomodulatory function and influence the induction, intensity, type and duration of the immune response.

6 The Place of Lipids in Age-related Changes in T-cell Signalling

Changing the extracellular and intracellular lipid composition influences cellular activation in many ways. The signalling events induced by lipids acting as antigens or as lipid mediators, are still under investigation. Nevertheless, there are several

pathological cases where T-cell dysfunctions are clearly associated with T-cell signalling defects, through the direct or indirect action of lipids. T-cell dysfunctions contribute to age-related immune dysfunction (immunosenescence). Altered TcR signal transduction is likely to play a role in this state of affairs (Pawelec et al. 2001; Fulop et al. 2005). Many signalling events, including the activation of the tyrosine kinase Lck, the adapter protein LAT, and the MAPKinase pathway are impaired in T-cells from elderly individuals. The final outcome of these alterations is a decrease in transcription factor translocation to the nucleus, reduced IL-2 production and reduced proliferative capacity, together contributing to age-related T-cell dysfunction and the global erosion adaptive immunity (Effros et al. 1997). Recent findings suggest that alterations of membrane raft properties and function are partly responsible for these defects (Larbi et al. 2004a). The cholesterol content, which is a critical parameter in membrane fluidity and the maintenance of raft structure and functionality, is increased in T-cells from normolipidemic elderly individuals. This may be an explanation for the impaired raft polarization at the stimulation site as well as decreased association of signalling molecules with rafts. The increased cholesterol content might be caused by alterations in cholesterol efflux or by the accumulation of oxidized cholesterol (Fielding et al. 2001). The free radical theory of aging postulates that increased production of reactive oxygen species (ROS) and reduced anti-oxidant machinery are critical factors in age-asssociated dysfunction (Harman 1969). Oxidized lipid levels are increased with age while anti-oxidant properties are decreased, helping to explain changes in membrane lipid composition, reduced T-cell signalling and increased dysfunction in immunosenescence (Stulnig et al. 1996; Jaouad et al. 2006).

As shown by the in vivo studies mentioned above, the intravenous infusion of lipids directly influences T-cell functions (Larbi et al. 2005). These experimental protocols aimed to mimic the effect of diet on immune function. Nutrition is an important factor in age-related immune dysfunction and in the regulation of T-cell signalling. As described above, lipids can influence T-cell signalling and functions and nutritional intake is the major source of circulating lipids. These effects are true for neonates as well as aged individuals (Calder et al. 2006; Field et al. 2006). Many clinical trials have aimed to assess the effect of nutrition on immune functions; however, these studies generally only investigated the effect of one nutrient. Because of the existence of nutrient to nutrient interactions as well as differences in the metabolism of different classes of lipids, entire nutritional intake must be considered (Garry et al. 2007). Further studies will be needed to establish the role of each lipid in the modulation of TcR signalling, T-cell functions and the global immune response.

7 Conclusions

T-cell function and signalling rely on membrane-dependent early events. Because the immediate environment lipid composition is directly influencing membrane composition this results in the modulation of T-cell signalling, function and the

immune response. Cholesterol is of major importance in this phenomenon because of its abundance in cell membranes as well as in the serum. Statins have significant protective effects against cardio-vascular diseases but also some unexpected effects on T-cell functions. One should consider this aspect when statin treatment is provided to immunocompromised individuals but would be beneficial to patient with autoimmune disease where T-cell hyperactivation is shown. There are some correlations between food intake and immune functions but more clinical studies will be needed to clearly identify candidates to improve immunity or to prevent senescence.

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Vaccination

Effect of Anti-influenza Vaccination on Immune System in the Elderly

Piotr Trzonkowski

Contents

Abstract: Prophylaxis with vaccines is of great importance in geriatrics as, apart from specific protection, it reduces the incidence of potentially fatal infectious complications and exacerbations of existing medical conditions. The level of postvaccination protection strongly depends on immune system and therefore markers of its condition may be used to predict the efficiency of vaccination. From the practical point of view, a link between some clinical features of the health status and condition of immune system are desirable as they allow to find the patients who may need additional care necessary to avoid possible complications, in case the vaccination did not protect them against the infection. This chapter reviews immune phenomena associated with anti-influenza vaccination. Humoral and cellular markers of the immunization efficiency are discussed in respect of health status of the elderly.

Keywords: Anti-influenza vaccination • The Senieur Protocol • Humoral response • T-cells • Antigen Presenting Cells

1 Introduction

There is consistent view among researchers and clinicians on the importance of vaccination against influenza in the elderly. Growing awareness of the benefits from the vaccination has recently resulted in the decrease of vaccination age threshold,

P. Trzonkowski (✉)
Laboratory of Experimental Transplantology
Department of Histology and Immunology
Medical University of Gdańsk
U1. Debinki 1, 80-211 Gdańsk, Poland
Tel.: 0048 58 3491430
Fax: 0048 58 3491436
E-mail: ptrzon@amg.gda.pl

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behind which the immunization is recommended for the entire population regardless of health status, from ≥ 65 to ≥ 50 years. In this age group the virus does not only cause influenza but, more important, it is responsible for secondary pneumonia and severe exacerbation of preexisting chronic conditions which in many cases may prove fatal. A meta-analysis of epidemiological studies revealed that the vaccination in this age group reduces the rate of hospitalization for influenza by 27–38% and reduces all-cause mortality by 45–56% (Vu T 2002; Nichol KL 2003). Importantly, the reduction in the rate of hospitalizations and deaths is comparable between healthy and frail elderly (Hak E 2002). Bearing in mind that around 80–90% of the mortality related to influenza and its complications occurs in the elderly, these results proof necessity for repeated annually prophylactic vaccination. The results also suggest that the vaccine might be a safe tool boosting immune system in the elderly.

Response of immune system to the challenge with anti-influenza vaccine depends on several variables. The most important are:

- Viral antigens in the vaccine (which directly reflects types of viral strains emerging in particular epidemic season)
- Type of vaccine administered
- Health status of immunized individuals.

Introducing the readers to the subject, it has to be stated that different, sometimes mutually exclusive, results of studies in the field may come from the fact that they were performed in different seasons, with different preparations of the vaccine available in particular seasons, and that the cohorts examined in different studies could be recruited and classified with the use of various criteria (Beyer WE 2006).

2 Virus and Vaccines

Influenza virus is RNA virus that belongs to *Orthomyxoviridae* family. Epidemic influenza in humans is caused by A and B types of viruses. Importantly, there is a possibility that human subjects may suffer from influenza caused by simultaneous infection with few types of the virus at the same time (Edwin D. Kilbourne 1951). Infection is the most severe in small children and in persons aged ≥ 65 years, which is the most dependent on immature immunity in the childhood and compromised immunity in the elderly (Couch RB 1994). Clinical efficiency of the vaccination defined as the percentage of subjects protected from the infection is strongly dependent on extreme variability of the virus. It is the reason why antigenicity of the vaccine has to be changed every season. New variants of influenza virus differing in their immunogenicity emerge as a result of antigenic "drift" and "shift". "Antigenic drift" is a gradual change of the viral genome dependent on point mutations which results in small antigenic modifications. This type of antigenic variability is relatively easy to follow by immune system primed with anti-influenza vaccine. On the other hand, "antigenic shift" is a substantial change in viral genome caused by exchange of

DNA fragments between animal and human strains of influenza virus. The "shift" is less common than "drift" but it results in radical change of viral antigenicity giving the virus potency to escape from efficient immune response. It makes the "shift" an important factor contributing to pandemic influenza. Interestingly, these completely modified viruses that cause pandemics are also capable of changing pattern of age-specific susceptibility to influenza. For example, second wave of the infection during the biggest pandemic in XX century, that is, during 'Spanish' influenza in 1918, was associated with the highest rate of mortality among young persons aged 20–34 years. In contrast, the lowest rate of deaths was noted among very elderly aged ≥ 70 years (HMSO 1920). To some extent, similar reversed pattern of mortality was also noted during 2 other severe pandemics in 1957–1958 and 1968–1969 (Payne AMM 1958; Schenbaum SC 1976). It is believed that the protection in the elderly was dependent on prior exposure to antigenically equivalent viruses earlier during ontogeny which left immune memory in the elderly. This is consistent with hypothesis of "antigenic recycling" which states that antigenic content of influenza viruses reemerges and the strains causing epidemics in particular seasons might be similar to those which circulated in previous era (Schenbaum SC 1976; Nguyen-Van-Tam JS 2003). Nowadays, WHO-based surveillance network of laboratories covering the entire globe identifies new potentially aggressive strains and updates the content of the vaccine to influenza strains challenging immune system in particular seasons. It also coordinates other necessary sanitary restrictions in order to prevent influenza pandemic. For example, first outbreak of avian influenza with previously unknown A/H5N1 virus took place in Hong Kong in 1997 but thanks to strict surveillance it has not caused pandemic as yet.

From the clinical point of view, there are 2 important viral proteins: neuraminidase and hemagglutinin. Immune response and therefore design of anti-influenza vaccines are based mainly on these 2 proteins. Currently, anti-influenza vaccine preparations contain antigens of three different strains that cover antigenicity of strains causing influenza in particular season. There are 2 main types of the vaccine available commercially: inactivated (intramuscular injection) and live attenuated (intranasal spray). However, only inactivated one is recommended for the elderly as it contains killed viruses. Inactivated vaccine is produced as split or subunit preparations. Split vaccine consists of whole disrupted viruses while subunit vaccine contains mainly 2 the most immunogenic proteins, neuraminidase and hemagglutinin, bound to a carrier.

Immunization with the vaccine as a source of alien antigens may produce mild symptoms of immune response which constitute majority of postvaccination adverse effects. Fever, malaise, myalgia, headache, etc. starting around 6-12h after immunization and lasting less than 2 days may occur in a small proportion of patients. More common effects are local soreness and swelling at the vaccination site (10-64% of patients). This type of effects is mainly related to the route of vaccine administration.

Quality of the vaccines has been continuously improving in order to satisfy rigorous criteria of efficiency and safety. Nowadays, inactivated vaccines are composed of only subvirion and purified surface antigens of the virus without its highly

Table 1 Virus and Vaccines

- Influenza— virus depends on:
  - antigenic drift—a gradual change of the viral genome dependent on point mutations
  - antigenic shift—(responsible for pandemics) a substantial change in viral genome caused by exchange of DNA fragments between animal and human strains
- Antigenic recycling—reemerging of the antigenic content of influenza virus strains which gives the elderly advantage of having immune memory during reexposure
- Neuraminidase—surface protein of influenza virus responsible for increased secretion and liquefaction of mucus covering epithelium of the respiratory tract; it allows for easier penetration of the virus within the respiratory tract; the protein also takes a part in budding of newly-synthesized virions from the surface of host cells and decreases innate immune response of the host to infected cells; applied as a content of anti-influenza vaccines due to high immunogenicity, it is also a target for the IInd generation of anti-influenza drugs
- Hemagglutinin—surface protein of influenza virus that binds its particles to sialic part of receptors expressed on the surface of respiratory tract epithelium which results in pinocytosis of the virus; it is also responsible for the generation of conglomerates of newly-synthesized virions underneath plasma membrane of host cells immediately before budding; used as a content of anti-influenza vaccines due to high immunogenicity
- Anti-influenza vaccine—trivalent vaccine containing antigens of three influenza virus strains recommended by WHO for particular epidemic season: 2 strains of Type A (H1N1 and H3N2) and 1 Type of strain B; 2 kinds of vaccine are routinely used:
  - inactivated—intramuscular injections produced as split or subunit preparations, **the only type recommended currently for the elderly**
  - live attenuated—produced as intranasal spray

pyrogenic lipid components. This way substantial decrease in systemic adverse effects after immunization was achieved. Severe adverse effects noted previously, such as increased rate of Guillain-Barre syndrome reported in the season 1976/1977 after administration of swine influenza vaccine (Schonberger LB 1979; Safranek TJ 1991), are nowadays much more limited with changed procedure of vaccine production. Currently, the vaccine is produced from viruses which are cultured in embryonated hens eggs and subsequently killed and purified (Gerdil C 2003). Unfortunately, it causes that persons hypersensitive to egg proteins can be immunized with these preparations only after desensitisation therapy and under physician's care. Apart from hen egg proteins, vaccines may be also contaminated with trace amounts of antibiotics used during vaccine manufacturing. Thus, although hypersensitive reactions occur rarely after influenza vaccination (Bierman CW 1977), the administration of the vaccine has to be always preceded by obtaining the history of existing allergies, in particular egg and drugs allergies. Also the possibility that mercury-containing vaccine preservative thiomersal might be allergic or toxic caused that the amount of this agent has been significantly reduced in modern vaccines and in some U.S. states thiomersal is completely banned (Centres for Disease Control and Prevention 2006). All these examples prove that not only influenza virus antigens but also other components of the vaccine are able to challenge immune system. Importantly, like in Guillain-Barre syndrome case, some components of the vaccines can substantially modify immune responses to viral antigens.

Table 2 Practical recommendations for anti-influenza vaccination in the elderly

- All adults age ≥ 50 years should be vaccinated regardless of their health status
  - egg allergy—patient must be desensitised first; in patients hypersensitive to egg prophylactic use of antiviral drugs should be considered instead
  - history of hypersensitivity after previous vaccinations—if patient is at high risk of influenza complications (aged ≥ 65 years), vaccination may be considered after appropriate allergy evaluation and desensitization; vaccine administration always under care of medical staff
  - allergy to antibiotics (also hypersensitivity to other components of the vaccine)—special attention should be taken, it is recommended to administer the vaccine under care of medical staff
  - patients with fever due to acute illness—immunization must be delayed 1–2 weeks since complete recovery
  - patients with the history of Guillain-Barre syndrome—should be vaccinated if aged ≥ 65 years; those 50–65 years old should be vaccinated only if at high risk group (suffer from chronic disorders of the pulmonary or cardiovascular systems; or require regular medical follow-up or hospitalization during the preceding year; or suffer from any condition that can compromise respiratory function in any way; or residents of nursing homes and other facilities that house patients who have chronic medical conditions)
- Single dose of trivalent inactivated vaccine, either split or subunit, is the only anti-influenza vaccine preparation recommended in the elderly
  [the preparation for particular season must be used, even though the antigenic content of the vaccine from previous season is the same); multi-dose vials may be used for vaccination in nursing homes and other long-term elderly-care facilities]
- The intramuscular route (in the deltoid muscle) is recommended; a needle length ≥2cm (1 inch) should be applied.
- Vaccine preparation should be stored and transported in +4°C until use
- Vaccination should take place in October–November, i.e., before epidemic season, in order to provide adequate serologic protection
- Earlier immunization (in September) should be avoided as post-vaccination specific immunity against influenza at this age may decrease below protection level before the peak of epidemic season
- Vaccination is still recommended after November for unvaccinated uninfected persons until the end of epidemic season (April)
- Treatment with IInd generation antiviral drugs* should be considered for infected patients:
  - zanamivir: 10mg (two inhalations) b.d. or
  - oseltamivir: 75mg b.d.

*Ist generation drugs are currently not recommended

- Inactivated vaccine can be administered within 4 weeks of any live or inactivated vaccines and vice versa, other vaccines can be administered after inactivated anti-influenza vaccine (the vaccine does not interfere with other vaccines)
- Inactivated vaccine can be administered simultaneously with pneumococcal polysaccharide vaccine
- Caregivers, medical staff, and any household contacts of the elderly should be also vaccinated

There are few important practical aspects which have to be highlighted here. First, as the vaccine contains killed viruses, it cannot cause influenza. Neither systemic nor local postvaccination symptoms can be considered as influenza infection but only as symptoms of immune response to exogenous antigens administered in the vaccine (in fact, these symptoms prove the vaccination works). It has to be explicitly

explained to the patients who very often identify these symptoms as “influenza” and neglect necessity of further annual vaccinations. Of note, several studies proved that those symptoms might be very much related to patient’s perception of the vaccination and not to real health postvaccination problems. In blinded trials the rate of adverse effects after anti-influenza immunization was equal to the rate of adverse effects after placebo injections (Margolis KL 1990; Govaert TM 1993). In our studies, patients, who reported side effects immediately after vaccination but were comprehensively informed about the procedure, did not remember these side effects six month later and were very positive to further vaccinations (Trzonkowski P 2003a). It proves both, mildness of the symptoms and necessity of proper education as a substantial part of vaccination prophylaxis. In many cases patients also report that they suffered from influenza despite the immunization. Nevertheless, not so often patients and their doctors verify the diagnosis with laboratory confirmation of the presence of influenza virus which is indispensable condition of such diagnosis (currently, commercially available rapid diagnostic tests can detect influenza viruses as quickly as in 30 minutes). It is known that more than one hundred viruses are able to produce symptoms of respiratory illnesses that, to great extent, may resemble influenza. Medical staff should inform the patient that anti-influenza vaccine protects from influenza virus and not from other respiratory viruses. The vaccine may increase general immunity but there is no guarantee that patients will not suffer from infections of respiratory tract other than influenza. Practically, good argument in favour of vaccination, which persuades patients to be immunized annually, is that although they still may be infected with other respiratory viruses, they are protected from often fatal complications of their chronic diseases. The argument for a very small proportion of those, who suffered from confirmed influenza infection despite vaccination, is that the symptoms of influenza are much milder after vaccination. Moreover, it has been proved that even unsuccessful first vaccination still increases the level of protection which will be gained with subsequent doses of the vaccine repeated annually in the future seasons (Keitel WA 1997).

3 Background of Immune Response to the Vaccine in the Elderly—from Clinic to Basic and Back

Understanding of immune response to anti-influenza vaccine in the elderly requires brief summary of some distinctive features of immune system in this age group. First of all, the elderly are very heterogeneous in their responses. Medical staff immunizing persons aged ≥ 65 years faces patients whose health histories are extremely diverse. The vast majority of these patients are frail elderly with a burden of multiple medical conditions and polypragmasy which are not neutral to immune system (Table 3). The other pole constitutes a small proportion of healthy elderly including those with limited number of relatively mild conditions. No doubt, immune system differs between these 2 groups and proof for that are centenarians whose immunity is now known to be different from “younger elderly” in many aspects (Franceschi C

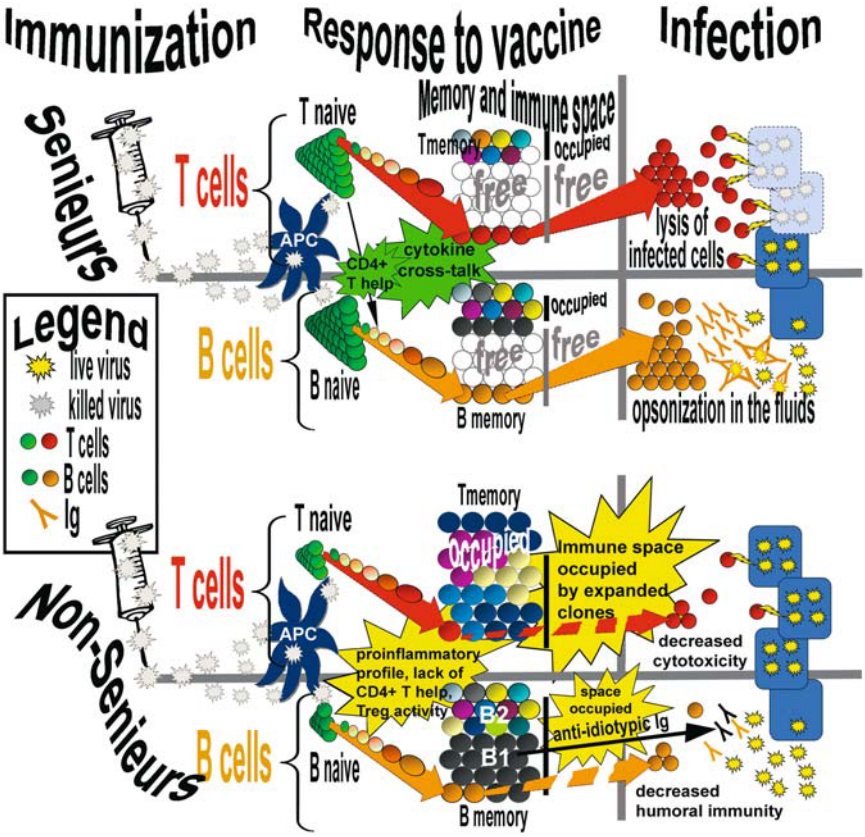

Fig. 1 Influenza vaccination affects differently the elderly differing in health status
Antigens of the killed viruses administrated in the vaccine are processed and presented via APC-cells. This presentation primes both naïve T-cells and naïve B-cells. Generation of memory cells is easy in „Senieurs“ (healthy individuals) whose naïve compartment is relatively big and few memory clones do not interfere with this process. „Non-Senieurs“ (unhealthy individuals) are characterized by much more limited number of naïve cells and expanded memory clones occupying immune space additionally prevent from the generation of immune memory to the vaccine antigens. Increased level of proinflammatory cytokines and increased activity of T-regulatory cells also contribute to the attrition of immune responses to the vaccine in these individuals. As a result, effective cellular and humoral responses to the infection with influenza virus are noted predominantly in „Senieurs“ who are capable of rapid and efficient expansion of virus-specific effector cells. These cells and their products fight against the virus in the body fluids thanks to the specific antibodies and within infected cells thanks to the cytotoxic effect. „Non-Senieurs“ are usually characterized by significantly reduced response during influenza infection which is too low to be effective enough. In addition, the negative effects present during the generation of immune memory to the vaccine in „Non-Senieurs“ are also present during the infection, which additionally limits the activity of already reduced number of virus-specific memory cells. Thus, despite immunization, „Non-Senieurs“ may suffer from the symptoms and signs of influenza.

1995). Thus, initial predictions of efficiency of the vaccination can be based on careful assessment of health status of particular patients. Classical approach in experimental immunosenescence studies on humans was initially offered by the "Senieur Protocol" criteria with some further amendments (Ligthart GJ 1984; Ligthart GJ 1994; Myśliwska J 1999; Castle S 2001). According to this concept it is possible to divide elderly population into frail "Non-Senieurs" and healthy and almost-healthy "Senieurs". Importantly, the majority of information necessary for the classification can be easily obtained based on physical examination, patient's history and very basic laboratory data. As it comes from original criteria, also confirmed by our results, the classification is more reliable when the patients are under continuous routine care and their records are systematically updated, rather than when the assessment is performed accidentally only for the needs of particular study. Applying the "Senieur Protocol" into clinical routine proved to be helpful in distinguishing between potentially good and bad elderly responders to anti-influenza vaccine, as "Senieurs" were mainly responders, while "Non-Senieurs" belonged mainly to nonresponders (Trzonkowski P 2003).

Interestingly, more recent concepts of immune health criteria based on recent achievements of basic science may somehow correspond to the "Senieur Protocol". It is important from the practical point of view, as relatively simple methods of the evaluation of immune system can be widely applied and serve every practitioner to distinguish patients at risk of poor response to the vaccination. Additional care, necessary to avoid possible complications, would be then given to such patients, should the vaccination did not protect them against the infection. Good example of such novel theory, which is a nice "bridge" merging clinical assessment of the "Senieur Protocol" with concepts of immunosenescence, is the hypothesis of "immune risk phenotype" (IRP), which, in brief, states that high $CD8^+$ and low $CD4^+$ T-cell numbers and poor T-cell proliferative responses are associated with increased morbidity and mortality in the elderly (Ferguson FG 1995; Wikby A 2005; Hadrup SR 2006). As the efficiency of vaccination is still routinely measured mainly as the titre of specific antibodies, it has to be noted that "risk" aging is also associated with impaired humoral immunity (Paganelli R 1992). Hypotheses of "inflammaging", infectious ageing, "telomere" aging, "shrunk immune space" may also well contribute to IRP and its clinical consequences. Namely, it has been known that some pathogens, with cytomegalovirus (CMV) as the most important among them, are able to drive continuously the activity of immune system in a very subtle level called sometimes "abortive infection" (Speir E 1994; Khaiboullina SF 2004). Effects of such continuous activation accumulate over years resulting in protracted proinflammatory status, so-called "inflammaging" (Franceschi C 2003). Inflammaging has been found mainly in frail elderly, therefore it is considered highly detrimental (Myśliwska J 1999; Franceschi C 2003). The activation of immune system driven primarily by CMV is substantially enhanced by inflammatory cytokines, which altogether results in the generation of expanded clones of $CD8^+$ T-cells recognizing CMV-like antigens, often referred as $CD28^-CD8^+$ T-cells (Pawelec G 2005). Despite effector phenotype, these $CD8^+$ T-cells are anergic, unproliferating, and terminally differentiated, which makes them functionally

ineffective (Boucher N 1998). Expansion of oligoclonal $CD28^-CD8^+$ T-cells under conditions of continuous stimulation, together with the involution of the thymus, results in so-called "shrinkage of the immune space" for new clones (Franceschi C 2000; Ouyang Q 2004). Oversimplifying, enlarged $CD28^-CD8^+$ T clones and low thymic output of naïve cells preclude substantially generation of new immunocompetent lymphocytes in response to the challenges with new antigens. This is also valid for the antigens administered in vaccines (Effros RB 2007). Importantly, subjects characterised by CMV carrier status, increased proinflammatory activity, and expansion of $CD8^+$ T clones possess characteristics of IRP and usually are classified as "Non-Senieurs" (Trzonkowski P 2003a). Thus, careful clinical evaluation of health status using predefined criteria, like those in the "Senieur Protocol", may be an important source of information about the condition of immune system and possible reactivity to the challenge with anti-influenza vaccine. Table 3 is an example of some traits of the "lifestyle" and some medical conditions which are more likely to place elderly subjects in the group of "Non-Senieurs" and, at the same time, highly probably make them nonresponders to anti-influenza immunization. Nonresponsiveness not only refers to the serological responses but also to those cellular. Moreover, "Non-Senieurs" are significantly more susceptible to all-cause respiratory tract infections during epidemic season than their healthy counterparts (Trzonkowski P 2003; Trzonkowski P 2003a; Trzonkowski P 2004). At this point, it should be stated that potentially low response to the vaccine predicted on the basis of clinical examination is not a reason for disqualifying from the immunization. Contrariwise, as the same conditions make those patients more susceptible to influenza infection and its complications, it is yet another argument for their immunization and additional care during the epidemic season. Moreover, in our experience, impairment in serological response could be improved in some "Non-Senieurs" with repetitive vaccination in subsequent seasons. Interestingly, patients, who improved their serological responses during second or third immunization with the vaccine were characterized by relatively well-preserved markers of cellular immunity such as high cytotoxicity of NK-cells, relatively high levels of Th1 cytokines (IL2 and IL15) and low proportion of $CD28^-CD8^+$ T-cells. In contrast to the first-vaccination responders, the second-vaccination responders were CMV carriers characterized by moderately elevated levels of proinflammatory cytokines (IL6 and TNFα) (Trzonkowski P 2003; Mysliwska J 2004).

The possibility that some subjects may improve their responses and escape from the 'frail' part of particular criteria was also found by the others. The problem is the fact that the groups distinguished within particular classification are still heterogeneous. For example, IRP was found to possess reversible characteristic as some aged individuals were proved to be able to move out from it (Wikby A 2006). In addition, in some studies health status did not prove to be associated with IRP (Nilsson BO 2003). Centenarians are also a good example of heterogeneity. It was found that environmental pressure selects more strictly men than women. Thus, centenarian men consists the minority of people aged ≥ 100 years but they are healthier than centenarian women, whose high proportion has suffer from many medical conditions since relatively young age and yet achieve this exceptional lifespan (Franceschi C

Table 3 Factors affecting response to anti-influenza vaccination in the elderly based on postvaccination titres of antihemagglutinins and antineuraminidases [142 elderly followed in 3 consecutive seasons 1999/2000, 2000/2001, and 2001/2002] [Trzonkowski P 2003a]

Increased response	Decreased response
Lifestyle	
• High education • Independent life • History of physical activity (≥ 12 months, regardless sport discipline) • Smoking [higher titres correlated with longer time of smoking and higher number of cigarettes smoked per day (!)]	• Life in long-term care facility • Sleep disturbances—often symptoms of depression (difficulties in falling asleep, early rousing from sleep, frequent rousing from sleep during the night, inverted wake/sleep rhythm, nightmares)
Diet	
Normal BMI, fatty and/or protein-enriched diet	Increased BMI (regardless hip/waist ratio), carbohydrate-enriched diet
Commonly used drugs administered in the elderly and found to affect response to the vaccine	
nitrates, ACE inhibitors, methyloxantins, antidepressants (SSRI),	β-adrenergic blockers, calcium channel blockers, neuroleptics, nonsteroid antiinflammatory drugs, statins, glicocorticosteroids, posttransplant immunosuppression, oncology drugs (various types)
Medical conditions found to affect response to the vaccine	
cholelithiasis,	coronary heart disease, chronic congestive heart insufficiency, arterial hypertension, diabetes mellitus type II, hypothyroidism, active peptic ulcer, chronic renal insufficiency, rheumatoid arthritis, spondyloarthritis, prostatic hyperplasia, neoplasms (also in remission), depression, dementia (regardless of etiology), anaemia (regardless of etiology but the most common anaemia affecting the results was microcytic one caused by iron deficiency), immunosuppression (HIV carriers, post-transplant immunosuppression)
Clinical characteristics according to the Senieur Protocol	
Senieurs	Non-Senieurs
Immune markers associated with aging	
	Immune Risk Phenotype (IRP), Status of CMV carrier, Increased level of proinflammatory factors

2000; Franceschi C 2003). The insight into genome is probably the way to systematize the classification. No doubt, selection for longevity and threshold for frailty are associated with genes. For example, there is underrepresentation of -174GG polymorphism of IL6 gene among centenarians (Bonafe M 2001). Bearing in mind that -174GG homozygotes are high IL6 producers and IL6 is one of the main reasons of inflammaging (Ferrucci L 1999; Harris TB 1999), it seems to be obvious that -174GG polymorphic variant is associated with early onset of frailty during ageing. Nevertheless, genomic approach is also far from clarity. For example, while

these genotypic data corresponds with occurrence of severe chronic conditions in the elderly, such as three-artery coronary stenosis (Myśliwska J 2006), it is not so well correlated with serological response to anti-influenza vaccine (Trzonkowski P, unpublished data). Nevertheless, despite the lack of serological correlation, vaccination against influenza was found to decrease the level of complications after coronary bypass grafting in -174GG homozygotes (Myśliwska J 2006a).

4 Humoral Response to Anti-influenza Vaccine

It is a long time since the disturbances in humoral response during aging were described for the first time (Thomsen O 1929). This compartment in the elderly not only is inefficient in response to the challenges with new foreign antigens but it is also dysfunctional generating increased responses to own antigens. Like in T-cells, oligoclonality may be an important reason of this dysfunctionality as the shrinkage of repertoire of lymphocyte B-cell receptor (BCR) and clonal expansions of B-cells responsible for various gammapathies were described during aging (LeMaoult J 1999; Li F 2001; Myśliwska J 2002; Weksler ME 2002). Paradoxical decrease in the number of circulating B-cells with concomitant increase in the level of IgG and IgA found in the elderly (Paganelli R 1992) can be easily explained by increased ratio of B1a $CD5^+$ cells to B2 $CD5^-$ cells and oligoclonality of the latter (Weksler ME 2002). It is B1a subset that produces autoantibodies, while B2-cells are responsible for the production of high-affinity alloantibodies including those after immunization with vaccines. Thus, decreased number and reduced clonality of B2-cells can be the reason of poor responsiveness to anti-influenza vaccine in the elderly. Moreover, half time of mature B2-cells increases with age concomitantly with reduced output of recent bone marrow emigrants, which may explain increased ratio of IgG and IgA to IgM in the elderly (Kline GH 1999). Apart from alterations in B-cells, some data from animal models suggest that decreased humoral responses with age may be attributed to altered activity of naïve $CD4^+$ T-cells from aged subjects (Eaton SM 2004). This impairment is mainly dependent on prolonged exposure of naïve T-cells to environmental agents at the periphery throughout life (Haynes L 2005). The most detrimental defect, which affects the function of this subset, is diminished production of IL2 (Linton PJ 1996).

Humoral response to anti-influenza vaccine, measured as the titre of antibodies directed against strain-specific hemagglutinins included in the vaccine, became the most common way of laboratory checking approved for the evaluation of the immunization efficiency (Pereira MS 1972; Potter CW 1979; Ligthart GJ 1998). While wide variety of parameters can be set based on specific antihemagglutinin (HI) antibody titres, the “gold standard” was introduced and accepted worldwide in order to unify inter-laboratory comparisons. In 1992, EU Committee for Proprietary Medicinal Products published officially guidelines on harmonization of requirements for influenza vaccines (Committee for Proprietary Medicinal Products 1992). Based on that, three the best parameters were chosen for the evaluation of vaccine efficiency on a population level:

- **Mean fold increase**—the postvaccination geometric mean titre (GMT) of specific HI antibodies
- **Seroprotection rate**—the percentage of patients with HI antibody titres ≥ 1:40 postvaccination
- **Seroconversion rate**—the percentage of patients with a fourfold increase in HI titres postvaccination

Without question such unification is welcomed, but one should remember that there are still some drawbacks behind it. The main limitation seems to be incomplete control of prevaccination state (Beyer WE 2004).

Similar evaluation of the efficiency of immunization on the level of particular subject should be based on **seroprotection** (HI titre ≥ 1:40—suggested in patients seronegative before vaccination) or **seroconversion** (fourfold increase in HI titre—more useful in patients seropositive before vaccination). As nowadays vaccines are trivalent, complete protection is achieved when the titres of all three antihemagglutinins reach this threshold (Trzonkowski P 2003), but in many studies response to 1 or 2 strains is treated as protection (Gardner EM 2001). Obviously, clinical protection measured as reduction in influenza morbidity depends on the strain which is predominant in particular epidemic season, therefore some discrepancies between laboratory and clinical results are possible (Keitel WA 1997). For example, a review of 31 experimental studies revealed that, as compared to 70–90% reduction in morbidity in younger subjects, the vaccination was efficient only in around 50% of immunized elderly (Goodwin K 2006). At the same time, protective titre of antibodies in the elderly can be as low as 28% versus 74% in younger groups (Bernstein ED 1998). Surprisingly, despite low level of specific antibodies, old patients may be still free from the infection. Odelin and colleagues described a group of 285 vaccinated elderly residents of long-term care facility among whose only 21% developed seroconversion but only one 95-years-old patient experienced influenza infection during epidemic (Odelin MF 1993). On the other hand, Gravenstein and colleagues described a cohort of 72 vaccinated elderly with confirmed influenza infection despite the fact that 60% of them were seroprotected and 31% achieved the titres as high as ≥1:640 (Gravenstein S 1994).

Protective titre of HI antibodies in general population is generated in the body around 7-14 days post-immunization and it is kept up to 12 months after administration of the vaccine (World Health Organisation 1993; Gross PA 1997; Brydak L 2002). The process is slightly delayed and protection does not cover 12 months in aged individuals. In Levine's studies protective response occurred in as many as 70% of immunized elderly patients but 28% of seroconversions was noted later than 4 weeks after vaccination. Moreover, protective titres disappeared in 68% of protected elderly within 6 months after vaccination (Levine M 1987). Similar delay and weaker humoral responses after anti-influenza vaccination in the elderly were noted when the level of local secretory IgA in the wash from nasal cavity was measured (Powers DC 1992). Despite continuous improvement in the vaccine manufacturing, these serological results seem to be still valid as they were confirmed with the use of modern trivalent inactivated vaccines (Trzonkowski P 2003a). Some studies suggest that improvement may be achieved with combined use of trivalent inactivated

vaccine with live, attenuated influenza vaccine administered 1 month later (Nakhin AN 1998). Two doses of anti-influenza vaccine administered ≥ 1month apart are already routine practice, mainly in children (Centres for Disease Control and Prevention 2006). The strategy of increased dose of the vaccine was also found effective in patients from risk groups (Palache AM 1993). Nevertheless, it has to be mentioned that some authors did not find any benefit from increased doses of the vaccine in general population of the elderly (Gross PA 1987; Levine M 1987; Palache AM 1993).

Diminished antibody response to the first anti-influenza vaccination in aged individuals can be explained by low availability of naïve B-cells that maintain primary response (De Bruijn IA 1999; Ikematsu H 2000; Mysliwska J 2004). On the other hand, some recall responses may be exaggerated as a result of molecular mimicry, that is, structural similarities between the structure of viral antigens in the vaccine and those which had challenged immune system in the past and left immune memory detected as antibodies. Thanks to the mimicry, these preexisting antibodies can cross-react with influenza virus antigens from the vaccine (Powers DC 1992). The mimicry seems to be of special importance when frail elderly population faces pandemic caused by influenza virus modified by "antigenic shift" (see: "antigenic recycling"). This way, B-cell memory may compensate for immune impairment and aged individuals can be surprisingly well protected during pandemic (Nguyen-Van-Tam JS 2003). To some extent, the same mechanism may improve humoral efficiency of repeated annual immunization including patients from risk groups, notably, when the same influenza strains are recommended as a vaccine content during subsequent epidemic seasons (Beyer WE 1996; Ikematsu H 1997; De Bruijn IA 1999; Ikematsu H 2000; Brydak LB 2000a; Mysliwska J 2004). Repeated annual immunization not only increases the titre of generated specific antibodies in consecutive seasons, but also improves their avidity (De Bruijn IA 1999). However, some studies question effectiveness of repeated vaccination showing that humoral response may be diminished if the immunized subject has preexisting titre of antihemagglutinins to viral strains administered in particular vaccine. This questionable effect, known as "Hoskins Paradox", may be age-specific as it was described in toddlers (Hoskins TW 1979) but excluded in the elderly (Beyer WE 1998; Beyer WE 1999; Gardner EM 2001). Despite these serological discrepancies, repeated annual vaccination is nowadays highly recommended as morbidity and mortality from influenza and its complications is substantially reduced in repeatedly immunized elderly, including those with low titre of postvaccination antibodies (Govaert TM 1994; Ahmed AE 1995).

5 Cellular Immunity to Anti-influenza Vaccine

Discrepancy between serological and clinical protection rates may be explained by simple fact that the immunization has to induce not only humoral but also cellular immune response to be effective. It is of special importance as the repopulation of influenza virus takes place inside infected cells and this stage of the disease is not

available to the antibodies. Otherwise, T- and NK-cells may still approach the virus inside infected cells. Both subsets are able to directly kill infected cells or stop replication of the virus by secreted interferons which inhibit synthesis of the viral proteins by host cells. Importance of cellular immunity during influenza infection in aged subjects was confirmed in many independent studies in humans and animals (Bender BS 1991; Mbawuike IN 1993), also with adoptive transfer of influenza-specific cytotoxic T-lymphocytes (CTL) to infected animals which resulted in complete eradication of the infection (Yap KL 1978).

There is growing evidence for cellular responses to the vaccine in humans. It has been revealed that anti-influenza vaccines are able to activate both T- and NK-cells (Gorse GJ 1997). The awareness of the contribution of cellular responses to post-vaccination immunity in the clinic raised not so long ago when the vaccination with interleukin 2 (IL2) as adjuvant in the vaccine gave promising results (Provinciali M 1994). As deficiency of IL2 is recognized as a one of the most important factors contributing to age-related immune impairment, its application in the elderly is of special interest (Effros RB 1983). The link between T-cells and vaccination was revealed in the research on Th1/Th2 cytokines after vaccination. Some strains were found to prime preferentially Th1 responses suggesting activation of cellular response, while the others stimulated mainly Th2 responses suggesting activation of humoral immunity (McElhaney JE 1998). Some studies in the elderly highlight the importance of Th1/Th2 paradigm, i.e. the theory that Th1 and Th2 responses are mutually exclusive and only one of them is leading when the body faces particular infection (Allen J 1997). Namely, Th1/Th2 imbalance towards Th2 cytokines was reported by many authors at this age (Huang YP 1992; Castle S 1997; Glaser R 2001; Haynes L 2002). Specifically during anti-influenza vaccination in the elderly, Th2 bias results in low activity of CTL (McElhaney JE 1998b). There are also reports showing reciprocal influence of both cellular and humoral axes of immunity. Namely, good cellular response after vaccination, measured as the activity of CTL to vaccine antigens, was concomitant with low titres of specific antibodies and vice versa, high titres of antibodies were associated with low CTL activity (Powers DC 1993). Interestingly, the direction of response after the vaccination may be modulated with the dose of the vaccine. Subjects who received single dose of the vaccine generated mainly Th1 response with high level of specific CTL, while those who received two doses were characterized by Th2-dependent immunity with predominant antibody response (McElhaney JE 2005). However, some experimental data proved that simultaneous boosting of Th1 and Th2 responses with vaccine antigens is required for efficient serological responses to the vaccine in humans (Bernstein ED 1998). Bearing in mind that nowadays vaccines are trivalent, it is highly probable that both humoral and cellular components of immune response may be triggered and detected at the same time after vaccination (McElhaney JE 1998; Mysliwska J 2004). It has been only recently confirmed in animal model that $CD4^+$ T-cells, main producers of Th1 and Th2 cytokines, are prerequisite for both antibody and cytotoxic responses to influenza virus antigens (Brown DM 2006). Initially, $CD4^+$ T-cells primed with viral antigens cooperate with B-cells in the generation of specific antibodies and, in the later stage of response, those primed $CD4^+$

T-cells acquire capability of perforin-dependent cytotoxic effect and become a part of effector arm of cellular immunity against the virus. These experiments proved possibility that humoral and cellular responses may occur together and both contribute to the protection against influenza. The balance between different parts of immune system is believed to be kept by "third subset" of $CD4^+$ T-cells, so-called T-regulatory cells (Treg cells—reviewed elsewhere in this book). These highly suppressive cells, rather than quench, regulate immune responses protecting the body against self-damage in autoimmune reactions. However, their accumulation with age might be responsible for oversuppression manifested as decreased efficiency of vaccines in the elderly. Indeed, the accumulation of Treg cells was revealed to be associated with poor humoral and cellular responses to the immunization with anti-influenza vaccine. The suppressive effect was even wider as Treg cells inhibited the cytotoxic activity of NK-cells stimulated with influenza vaccine antigens (Trzonkowski P 2003a).

There is growing evidence for superiority of cellular markers above those humoral in prediction of vaccine efficiency. Some studies in humans revealed that, apart from the above mentioned perforin content, also production of granzymes after stimulation with vaccine antigens is associated with clinical protection to influenza infection. It was found that secretion of this marker of cytotoxic response was a better predictor of vaccine efficiency than the titre of specific antibodies (McElhaney JE 1998a; McElhaney JE 2006). Apart from granzymes, IFNγ secretion and number of influenza-specific memory T-cells were found to be better postvaccination predictors of protection against influenza infection than production of specific antibodies (Trzonkowski P 2003a; Deng Y 2004). While these results are promising, it has to be highlighted that the majority of them were obtained in small, not controlled studies. Thus, their importance must be confirmed in larger blinded trials before markers of cellular immunity may be applied routinely.

To some extent, the reason why cellular markers are not routine parameters of vaccine efficiency may come from the fact that sensitive methods of measurement of cellular responses, which might be applied in the clinical routine, have been available for around last ten years only. Among them, ELISPOT assay seems to be of special interest. It is the assay that allows for the measurement of exact number of cells producing particular protein (Altman JD 1996). In the studies of cellular response after the vaccination, the application of ELISPOT measuring IFNγ production after the stimulation with vaccine antigens is probably the most common (Deng Y 2004; McElhaney JE 2006). Sensitivity of the method is higher than flow cytometric analysis of intracellular cytokines or methods analyzing extracellular concentration of the cytokines in blood, supernatants, urine etc. For example, the method allows for detection of antigen-specific cells in samples taken from patients after peripheral depletion of mature lymphocytes. The frequency of lymphocytes in such individuals is often as low as below 1 cell/μL and yet the ELISPOT allows for detection of antigen-specific cells (Trzonkowski P 2006). Another interesting method on the clinical horizon is the tetramer staining, which is the measurement of $CD8^+$ T-cells with a given specificity. The tetramer is a complex of soluble MHC Class I receptors conjugated with a given peptide, for example, viral antigen from

the vaccine. Tetramer is also conjugated with fluorescent dye in order to be visualized. While incubated with blood, the complex is bound only by $CD8^+$ T-cells that express TCR receptors recognizing the peptide conjugated to the tetramer (Doherty P 1998; Murali-Krishna K 1998). Thus, the tetramer "stains" antigen-specific cells which can be subsequently counted using sensitive fluorescence reader, usually flow cytometer. Sensitivity of the method is very high as it is possible to detect specific cells in the mixtures where they consist less than 0.1% of cells. It means that, in theory, it is possible to detect as few as a single antigen-specific cell in the sample. The main disadvantage of the method is that it requires typing of MHC as particular tetramers may be applied only if they are matched with MHC molecules of particular patient. The new perspectives of this method are recently introduced MHC soluble complexes Class II which allow for the studies with $CD4^+$ T-cells.

It seems that $CD8^+$ T-cells are central and the most essential cells for cellular immunity after anti-influenza immunization. Their activity as CTL—secretion of granzymes, perforin and IFNγ—is crucial during effector stage of immune response, i.e., during elimination/killing of virus-infected cells and therefore for the clinical outcome of the immunization (McElhaney JE 2001). However, the repertoire of naïve $CD8^+$ T-cells is severely restricted with age due to decreasing thymic output. In addition, impaired function of $CD4^+$ T-cells, in particular their shift towards Th2 responses associated with frail aging (Castle S 1997), additionally contributes to weak cellular immunity in the elderly (Effros RB 2003).

Important part of response after immunization is the generation of T-cell immune memory. Like the switch from IgM to IgG in case of humoral immunity, efficient immunization is also associated with the transition from naïve to memory T-cells. As compared to animal studies, reports on this phenomenon in aged human subjects after anti-influenza vaccination are surprisingly scarce. Phenotypic analysis of the percentage changes in isoforms of CD45 receptor on T-cells revealed that the vaccination is associated with a decrease in the proportion of naïve $CD45RA^+CD45RO^-$ T-cells (McElhaney JE 1993). Deficit in the number of naïve T-cells might be the most important reason of inefficiency of the vaccination with age as Murasko proved in animal model that per-cell activity of influenza-specific T-cells does not differ between young and old subjects (Po JL 2002). With decreasing number of naïve $CD45RA^+CD45RO^-$ T-cells, the level of double-negative $CD45RA^-CD45RO^-$ T-cells was found to be increased in the peripheral blood of vaccinated subjects (McElhaney JE 1993). The $CD45RA^-CD45RO^-$ phenotype was described as atypical memory T cells, most probably because naïve $CD45RA^+$ / memory $CD45RO^+$ paradigm was still valid these days. It is now known that the expression of CD45RO receptor is unstable and it is more a marker of activation than memory as memory cells may not express it (Wills MR 1999). Thus, phenotypic definition of memory T-cells is more reliable when based on the absence of CD45RA receptor rather than on the expression of CD45RO receptor. Current understanding of naïve and memory compartments is that naïve T-cells transform upon challenge into 2 memory subsets, central memory and effector memory (Sallusto F 1999). Such discernment is of special interest among the elderly as the proportion of memory cells increases with age at the expense of naïve cells and the level of particular subsets of memory

cells might be associated with health status (Saule P 2006). T central memory cells (Tcm) express 2 important receptors, CCR7 and CD62L, which enable them to traffic into peripheral lymphoid tissue. Tcm are characterized by intermediate effector function and still possess capability of proliferation. On the other hand, T-effector memory cells (Tem) are triple-negative $CCR7^{-}CD62L^{-}CD45RA^{-}$ cells which makes their trafficking into lymphoid tissue impossible. Thus, they are predominant in peripheral blood and traffic to nonlymphoid tissues. Tem possess high effector potential and usually they are close to terminal differentiation with extreme $CCR7^{-}CD62L^{-}CD45RA^{+}$ T subset present mainly in $CD28^{-}CD8^{+}$ T-cells (Saule P 2006; Trzonkowski P 2006). $CD3^{+}CD28^{-}CD8^{+}CCR7^{-}CD62L^{-}CD45RA^{+}$ phenotype, often called TemRA cells, might be treated as detailed phenotype of $CD28^{-}CD8^{+}$ T-cells, that is, the subset accused of compromised immunity in the elderly (Pawelec G 2005; Effros RB 2007).

Accumulation of $CD28^{-}CD8^{+}$ T-cells, which "shrank immune space" for new antigenic challenges, was revealed to be associated with low responsiveness to anti-influenza immunization in the elderly (Goronzy JJ 2001) Their clonal expansions were then widely recognized as a burden associated with poor outcome of anti-influenza vaccination (Saurwein-Teissl M 2002; Trzonkowski P 2003). Nevertheless, some reports have revised our understanding of this population. It appears that homeostasis of $CD8^{+}$ T-cells, including $CD28^{-}CD8^{+}$ T-cells, is highly dependent on cytokines (Ku C 2001). Weng's study confirmed that it is possible to restore activity of $CD28^{-}CD8^{+}$ T-cells in the presence of IL15 (Chiu WK 2006). Of note, poor outcome of anti-influenza vaccination in the elderly was associated with low availability of IL15 and accumulation of anergic $CD28^{-}CD8^{+}$ T-cells (Trzonkowski P 2003a; Mysliwska J 2004). Thus, like in the case of IL2, IL15 supplementation might be considered as an adjuvant therapy during immunization of frail elderly. It might be also possible that $CD28^{-}CD8^{+}$ T-cells are heterogeneous and a proportion of them is characterized by reversible anergy. Indeed, it is possible to distinguish some subsets within this phenotype (Filaci G 2002). At this point, it has to be mentioned that healthy elderly, qualified as the "Senieurs" according to the "Senieur Protocol", are usually characterized by relatively high level of $CD8^{+}$ T-memory cells with less differentiated $CD62L^{+}$ Tcm phenotype and it corresponds with their good response to anti-influenza vaccine (Trzonkowski P 2003a). Interestingly, such T-memory cells expressing CD62L receptor may maintain not only cellular but also humoral memory responses (Schwaiger S 2003). It seems that accurate ratio of central to effector memory cells is prerequisite for the maintenance of anti-influenza memory response within $CD4^{+}$ T-cells after vaccination. Capability of transformation from Tcm into Tem cells in $CD4^{+}$ T subset was found to be a condition for long-term immune memory after anti-influenza immunization (Kang I 2004; Roberts AD 2005). Unfortunately, the transition is dependent on IL7, which level decreases with age concomitantly with thymus involution (Andrew D 2001). Thus, high proportion of the elderly is characterized by the accumulation of Tcm and deficit of Tem due to low availability of IL7. As a result, regardless of good initial responses immediately after the vaccination, $CD4^{+}$ T-cell recall responses three months after the exposure to vaccine antigens are much lower than those noted in the young (Kang I 2004).

As already mentioned, important difference between B- and T-cell responses, which suggests deeper impairment of cellular response with age, is that repeated annually vaccination corrects for low effectiveness of humoral response but not for cellular one. It was found in several studies that some cellular responses do not improve (Trzonkowski P 2003a), or may even worsen (McElhaney JE 1996), in consecutive vaccination seasons.

Apart from T-cell responses, NK-cells also contribute to cellular immune surveillance. High activity of these cells with age has been recognized as a compensation for "immune attrition" of T-cells in the elderly (Myśliwska J 1992; Franceschi C 2000). When T-cells are compromised, NK-cells are the last line of defense against intracellular pathogens. A fall in their number or activity predicts increased morbidity and mortality in the elderly (Levy SM 1991; Ogata K 2001). Their activation was also described after anti-influenza vaccination (Shapiro JM 1990; Myśliwski A 2001). Importance of this effect should not be neglected as NK-mediated cytotoxicity was confirmed to be essential for clearance of the virus in animal studies (Bot A 1996). The most important mechanism of NK-dependent clearance is associated with recognition of viral proteins by NK-cell activating receptor NKp46. The receptor is recognized as crucial in signalling that eventually triggers NK-dependent lysis of host cells infected by influenza virus (Arnon TI 2001; Mandelboim O 2001). Lack of this signal was found to cause lethal influenza in animal model (Gazit R 2006). In addition, during respiratory tract infections NK-cells secret copious amounts of IFNγ which augment CD8+ T-cell responses (Hussell T 1998). Important feature of NK-cells is that once activated by viral antigens or vaccine, they can keep the activation state for a long time, up to 20–30 days (Hussell T 1998; Mysliwska J 2004). As the clearance of the virus usually takes not so long, it raises the question on the reasons of this prolonged overactivity of NK-cells. The explana-

Table 4 Immune response to anti-influenza vaccine

Humoral response	Cellular response
Parameters	
- titer of specific antihemagglutinin (HI) antibodies - mean fold increase of HI antibodies - seroprotection (HI titre ≥ 1:40) - seroconversion (4-fold increase in HI titre)	- level of perforin - level of granzymes - secretion of IFNγ - number of influenza-specific T-cells
Disturbances in aging that affect efficiency of the vaccination	
- Increased B1/B2 cells ratio - Increased ratio of IgG and IgA to IgM - Impaired primary response and oligoclonal recall response - Impaired function of Th cells	- Th1/Th2 imbalance towards Th2 cytokines - Decreased thymic output of T-cells - Low level of naïve $CD8^+$ T-cells - Oligoclonality and terminal differentiation of $CD8^+$ T-cells (high level of $CD28^-CD8^+$ effector memory T-cells) - Low activity of NK-cells - Accumulation of T-regulatory cells
Repeated annually vaccination	
– improves the response	– does not improve the response

tion may come from recent report that proves involvement of NK-cells in immune memory and questions "handbook dogma" that these cells belong exclusively to innate immunity (O'Leary JG 2006). The other explanation is linked to cytokine milieu. Namely, it has been found that successful anti-influenza vaccination was associated with secretion of cytokines that induce NK-cell activity. Elevated levels of Th1 cytokines, such as IL2, IL12 and, IL15, were detected in "Senieurs" as long as one month postvaccination (Mysliwska J 2004). Thus, correlation between good outcomes of the immunization and high activity of NK-cells might be treated as yet another example of the importance of NK-cells in healthy ageing. Like the activity of T-cells, the activity of NK-cells does not improve with repeated vaccinations and can be attributed to health status of particular subject (Mysliwska J 2004).

6 Antigen Presentation—Missing Part of Immunization Strategy

Surprisingly, little is known about antigen presenting cells (APC) in successful anti-influenza vaccination in aged individuals. Some reports suggested that low number of alveolar macrophages in aged individuals might be a risk factor for respiratory tract infections (Zissel G 1999). On the other hand, the presence of macrophages was recognized as an obstacle in effective immunization and their depletion improved the results (Garg M 1996). However, it seems that the number differences might be less important than altered function of monocytes and macrophages. It has been revealed that several levels of the process of antigen presentation are affected in macrophages from aged individuals. Their capability of "sensing of danger" was found to be diminished in the elderly due to decreased expression of TLR receptors—the most important group of receptors responsible for sensing of microbes (Renshaw M 2002). Macrophages from aged mice were also revealed to possess decreased phagocytic and endocytic activities (De La Fuente M 1985; De la Fuente M 2000; Videla LA 2001) and their capability of migration to the site of inflammation was also reduced (Fietta A 1993; Ashcroft GS 1998). Apart from impairment in antigen capturing and chemotaxis, also antigen presentation might be affected during ageing. Macrophages from the elderly, as compared to the young, were found to express lower levels of CD80 receptor—an important element of second signal during antigen presentation to T-cells. There was also a correlation between the percentage of cells expressing CD80 receptor and effectiveness of anti-influenza vaccination (van Duin D 2007). The majority of those dysfunctionalities might be linked to hyperactivation of macrophages, a part of wider phenomenon of inflammaging. These cells are well-known source of proinflammatory agents and, at the same time, their production of chemokines is substantially reduced in the elderly (Ershler WB 1993; O'Mahony L 1998; Swift ME 2001). Altogether, inflammageing resembles chronic infections with intracellular pathogens, where macrophages are highly activated but anergic in a prolonged way (Chacon-Salinas R 2005; Lay G 2007). Apart from compromised immunity, including insufficient vaccination outcomes, such

chronic activation of macrophages may have an impact on wider range of aspects of frailty in aging. For example, in 3-years-lasting follow-up, patients classified as "Non-Senieurs", were in many cases found to be characterized by monocytosis and finally their response to anti-influenza vaccination was insufficient (Trzonkowski P 2003a). Of note, monocytosis in this study was associated with idiopathic microcytic anemia. Some previous studies explained association between low effectiveness of anti-influenza vaccination, anaemia and low level of iron by nutritional status of the elderly (Fulop T Jr 1999). Nevertheless, this phenomenon might be also explained by the fact that activated monocytes and macrophages present in inflammageing are overefficient stores of iron, which makes this microelement unavailable for erythropoiesis. The overactivity of macrophages might be also responsible for failed attempts of improvement of anti-influenza vaccination with iron supplementation (Crogan NL 2005). Complexity of the links between monocyte activity, health status and final outcome of anti-influenza vaccination proves necessity of further investigation in this area. Nevertheless, some basic knowledge, such as monocytosis or features of microcytic anemia in the blood count, can be obtained as simply as in a family doctor clinic and might help in presumptive prediction of the immunization results.

Dendritic cells (DC) are the most potent subset of APC. This population seems to be especially valuable in the generation of immunity after anti-influenza vaccination. DC were found to be superior to monocytes in the presentation of influenza antigens from the vaccine as they were the only APC capable of elucidating robust proliferative and effector responses of senescent T-cells (Lunga TL 2000). The same group showed that the generation and maturation of myeloid DC in vitro did not differ between young and elderly and responsiveness of DC, specifically to anti-influenza vaccine, was unimpaired in old age (Saurwein-Teissl M 1998a). Studies on currently recommended in the elderly inactivated vaccine revealed that the vaccine was capable of efficient stimulation of DC maturation. In addition, the vaccine antigens triggered secretion of cytokines in both DC and subsequently primed T-cells (Saurwein-Teissl M 1998). Indeed, other authors also confirmed relatively good condition of DC in aging. The proportion of DC and their expression of pattern recognition receptors (PRRs—the group of surface receptors that sense microbial antigens) including TLR receptors were found to be almost unaffected in the elderly (Agrawal A 2007b). Altogether, this data implied that boosting of DC in vivo should be one of the targets of anti-influenza vaccination. However, some other reports suggested that some features of DC are different between young and elderly individuals. The impairments found in DC subsets seem to be more profound in frail elderly. First of all, it is possible that density of DC in nonlymphoid tissues, such as skin and oral mucosa, is decreased in the elderly (Thiers BH 1984; Choi KL 1987; Rittman BR 1987). Lymphoid tissue is also affected as follicular DC in the elderly were revealed to induce fewer and smaller germinal centres (Szakal AK 1988). There are also reports on their impaired function in the elderly (Villadsen JH 1987). Like in the case of monocytes, altered function of DC was described in the elderly on many levels. DC from the elderly are less efficient in antigen trapping, its processing and transport. Thus, their ability to present antigens is diminished.

Known reasons of this impairment are low surface expression of FcR receptors on DC and decreased activation of the PI3K-signalling pathway in their cytoplasm (Szakal AK 1988; Wick G 1997; Sato H 1998; Aydar Y 2004; Agrawal A 2007). To some extent, impaired antigen presentation might be also dependent on apoptosis of myeloid cells at this age. Namely, it is widely accepted that apoptotic bodies from monocytes and macrophages, which died upon stimulation with influenza antigens, can be caught by DC and then vaccine antigens from those macrophages may be effectively presented by DC. General view on apoptosis is that the cells in the elderly are prone to undergo this process easier than their counterparts in the young (Aggarwal S 1998; Aggarwal S 1999). However, detailed analysis revealed that this is only valid for the group of healthy elderly, while cells from those frail are resistant to apoptosis (Szmit E 2002; Trzonkowski P 2003a). Altogether, it clearly suggests that the antigenic load of DC dependent on decreased antigen trapping, impaired antigen transport, processing and low intake of apoptotic bodies might be impaired in elderly nonresponders. It means that each and every patient receives the same dose of the vaccine but the amount of vaccine antigens which is presented and takes part in priming of T and B cells is diminished in nonresponders and therefore the response of nonresponders is insufficient. Obvious solution to that problem would be increased amount of influenza antigens administered in the vaccine or multiple doses of vaccine given to nonresponders. While these manipulations are currently applied in children, they have not found approval in the elderly due to conflicting results of experimental trials (Gross PA 1987; Levine M 1987; Palache AM 1993).

Table 5 Presentation of influenza vaccine antigens *) **)

Monocytes/Macrophages	**Dendritic cells (DC)**
Parameters	
- number in peripheral blood and tissues - phagocytic activity - antigen presentation - secretion of cytokines - chemotaxis - oxidative burst - expression of surface markers	- number and proportion of myeloid/lyphoid DC in peripheral blood and tissues - chemotaxis - antigen uptake - antigen presentation - secretion of cytokines - stimulation of T-cells - expression of pattern recognition receptors (PRR)
Disturbances in aging that affect efficiency of the vaccination	
- decreased phagocytic and endocytic activities - decreased expression of TLR receptors - increased secretion of proinflammatory agents - impaired secretion of chemokines - impaired apoptosis	- relatively well preserved function - decreased density of DC in peripheral tissues - decreased capability of antigen presentation

* for B-cells please refer to Table 4

** during influenza infection antigens of influenza virus are also presented by nonprofessional APC; epithelial cells lining upper respiratory tract and, mainly in case of complications, epithelium of lower respiratory tract, neurons, heart and, skeletal muscle fibers

7 Perspectives

Currently, there are several approaches to increase effectiveness of anti-influenza vaccination. Increased immunogenicity of the vaccine can be achieved by adding adjuvants. For example, promising results with dehydroepiandrosterone (DHEA) in animals prompted clinicians for its application in humans. Surprisingly, the results were inconsistent and some of the studies revealed that DHEA may even decrease the efficiency of vaccination (Araneo B 1995; Danenberg HD 1997; Ben-Yehuda A 1998). Melatonin, another hormone which level decreases with age, was also suggested as efficient adjuvant of the vaccine (Pierpaoli W 1987). Bearing in mind that melatonin is responsible for circadian rhythms and sleep disturbances are associated with low effectiveness of the vaccination (Trzonkowski P 2003a), further studies with this hormone should be granted. Another approach is based on simultaneous administration of cytokines, such as IL2 or GM-CSF, in combination with vaccines (Provinciali M 1994; Babai I 2001). Cyclooxygenase inhibitors (NSAID), commonly used drugs, were also suggested as adjuvants. Nevertheless, simultaneous administration of NSAID with anti-influenza vaccine gave inconsistent results. Some studies proved their effectiveness, while other works did not find any differences or revealed low efficiency of the vaccination in NSAID treated group (Gross PA 1994; Hsia J 1994; Trzonkowski P 2003a). Supplementation with trace elements was also postulated as a tool of vaccination improvement (Girodon F 1999). Recent reports on the role of zinc in immune responses in the elderly seem to be the most promising gate in this area (DelaRosa O 2006). Synthetic adjuvants are also in the clinical use. Licensed in Europe FluAD® vaccine is the preparation based on MF59 emulsion (contains squalene, Tween 80, sorbitan oleate) which, as compared to the conventional trivalent inactivated vaccine, generated 1.5 fold higher titres of specific HI antibodies in the elderly (Podda A 2001). However, it caused more adverse effects. ISCOM technology is another strategy. It is based on the use of virosomes, viral envelopes without genetic material, which were proved to prime cellular immunity (Rimmelzwaan GF et al. 2001). Inflexal-V® is the first preparation of virosome-based vaccine licensed in Europe. Immunostimulating complexes called iscoms are very much close to virosomes. These are mixtures of viral antigens with cholesterol, saponine and phosphatidyl choline which structure resembles virion envelope with viral antigens. The complexes were found to induce both humoral and cellular immunity in animal models. However, their use in humans is still questionable as high titres of specific antibodies detected 1 week postvaccination disappeared quickly and 4 weeks after the vaccination no difference with conventional vaccines was noted (Rimmelzwaan GF et al. 2000).

Apart from adjuvants, other vaccine antigens are tried to be applied in order to increase vaccine immunogenicity. NP and M2 proteins, which in theory are much more conservative than hemaglutinin or neuraminidase, were found to generate protection against wide variety of viruses in animal models (Neirynck S 1999). Their disadvantage was that they did not protect from influenza B virus (Ulmer JB 2002). There are attempts to deliver NP and M2 by DNA immunization, in order to

increase their immunogenicity. Surprisingly, although such vaccine was proved to elicit both humoral and cellular responses, animals immunized with this protocol developed fatal influenza (Heinen PP 2002).

Alternative routes of vaccine delivery are also tested. A natural target is the skin, which strong immunogenicity is dependent on the presence of specific subset of dendritic cells, so-called Langerhans cells. A kind of revolver that shoots a powder containing viral antigens was designed to deliver the antigens to these cells (Chen D et al. 2001). Another approach is to deliver the vaccine directly to mucosa. Intranasal spray containing live attenuated virus FluMist® is currently licensed in USA for patients aged 5–49 years. Similar technique using novel preparations of inactivated vaccine is tested in several studies but the results are not yet convincing (Boyce TG 2000; Plante M 2001). For example, already licensed intranasal virosome-based preparation NasalFlu® was withdrawn as its administration was associated with increased number of neurological complication—Bell's palsy (Palese G 2002).

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Immunosenescence Modulation by Vaccination

Janet E. McElhaney

Contents

Abstract: A decline in immune function is a hallmark of aging that leads to complicated illness from a variety of infectious diseases, cancer and other immune-mediated disorders, and may limit the ability to appropriately respond to vaccination. How vaccines might alter the senescent immune response and what are the immune correlates of protection will be addressed from the perspective of 1) stimulating a previously primed response as in the case of vaccines for seasonal influenza and herpes zoster, 2) priming the response to novel antigens such as pandemic influenza or other viruses, 3) vaccination against bacterial pathogens such as pneumococcus, and 4) altering the immune response to an endogenous protein as in the case of a vaccine against Alzheimer's disease. In spite of the often limited efficacy of vaccines for older adults, influenza vaccination remains the only cost-saving medical intervention in this population. Thus, considerable opportunity exists to improve current vaccines and develop new vaccines as a preventive approach to a variety of diseases in older adults. Strategies for selecting appropriate immunologic targets for new vaccine development and evaluating how vaccines may alter the senescent immune response

J. E. McElhaney (✉)
9B Providence, St. Paul's Hospital
1081 Burrard Street
Vancouver, BC
Tel: (604) 806-9153
Fax: (604) 806-8390
E-mail: janet.mcelhaney@ubc.ca

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in terms of potential benefits and risks in the preclinical and clinical trial phases of vaccine development will be discussed.

Keywords: Vaccination • Correlates of protection • Helper T-cells • Cytotoxic T-lymphocytes • Antibodies • Cytokines • Granzyme B • Influenza • Herpes zoster • Pneumococcus • Alzheimer's disease

1 Introduction

This review will focus on vaccine preventable diseases and the effect of vaccination on the senescent immune response to specific pathogens, observed in community-dwelling older adults and relevant experiments in animal models. It is important to distinguish these studies from those in older people in the nursing home setting who represent a small minority of the population age 65 years and older; multiple chronic diseases have already impacted on morbidity and disability and immune function may no longer be representative of the senescent phenotype. As a population, the majority of older adults experience "usual aging" where independence is maintained in the community but risk for complicated illness is associated with one or more underlying chronic diseases. From a public health perspective, usual aging older adults are the largest segment of the population age 65 years and older and should be the target population for new vaccine development; the goal is to compress morbidity to the extremes of life—"adding life to years".

A second focus of this review is to highlight the challenges in vaccine development for older adults and how vaccines may interact with the senescent immune response in ways that are not predictable using standard techniques such as antibody titres to evaluate potential efficacy. The goal of vaccination in this population should be clinical protection rather than sterilizing immunity; sterilizing immunity is predicted by antibody titres whereas estimates of vaccine efficacy with respect to clinical protection require an evaluation of both humoral and cell-mediated immune responses to a vaccine or the relevant pathogen. In addition, vaccines need to be tested in usual aging older adults who experience a variety of common medical conditions and related medications, and mental and psychosocial health issues, all of which may interact with functional independence. While studies of healthy older adults will help us to understand the effect of aging on the immune response, translating this research to vaccine preventable disease and improved health outcomes in the 65 and older population requires the identification of "modifiable risk" at all levels of innate and adaptive immune function.

2 Immune Senescence: Stimulating a Primed Response

2.1 Influenza Vaccination

2.1.1 Influenza, the Most Vaccine Preventable Disease in Older Adults

Influenza is foremost among all infectious diseases in terms of risk for serious complications and death in older adults and is the most vaccine preventable disease in this population. At least 36, 000 deaths and more than 100, 000 hospitalizations from respiratory and cardiovascular complications of influenza occur annually in the United States [1, 2]. In spite of only 40–60% efficacy in older adults [3], current influenza vaccination programs are cost-effective in older people and even cost saving in developed countries due to the 30–40% reduction in influenza-related hospitalizations [4, 5]. The fact that these vaccines also prevent complications of influenza (pneumonia, heart attacks, strokes and exacerbations of congestive heart failure) provides an even greater incentive to increase the use of existing vaccines and develop new vaccines that are targeted to improve the senescent immune response [6, 7]. However, a limited understanding of the immune mechanisms that underlie the increased risk for complicated illness and decline in the response to vaccination in this population, pose a significant challenge to new vaccine development.

2.1.2 Influenza Virus Stimulates both Humoral and Cell-mediated Immunity

The effect of influenza vaccination on the senescent immune response is best understood from the perspective of the adaptive immune response to influenza and how this may be altered through vaccination. Influenza vaccine is the most studied vaccine in older adults and well understood in terms of the potential immunologic determinants of clinical protection in this population. Thus, the response to this virus in the context of age-related changes in the adaptive immune system, will be discussed in significant detail as an example of what we might anticipate in terms of other potentially vaccine preventable diseases in older people.

Influenza virus stimulates an antiviral response in bone-marrow-derived lymphocytes (B-cells), monocytes and thymus-derived lymphocytes (T-cells) resulting in humoral and cell-mediated immunity, respectively. However, the effectiveness of this stimulus depends on the presentation of viral peptides to the T-lymphocytes There are two main cell types within the T-lymphocyte population, helper T-cells and cytotoxic T-cells. Helper T-cells are further sub-typed (according to the cytokines they produce) as T helper type 1 (T_h1), T helper type 2 (T_h2), T helper type 3 or regulatory T-cells (T_h3/Treg), and T helper type 17 (T_h17) cells. The response to influenza virus in adult populations is the result of restimulation of a previously primed response through exposure to natural infection or prior vaccination. Virus-activated T-cells, through a variety of cytokine mediators, stimulate B-cells to differentiate and

produce antibodies that are specific for the strains of virus contained in the vaccine [8]. These specific antibodies bind to the surface glycoproteins (haemagglutinin [HA] and neuraminidase [NA]), to neutralize the viral particle. The peptide sequences on the outer surfaces of HA and NA change as a result of high mutation rates in the influenza virus and selective pressure by the immune system against the native virus, a phenomenon known as antigenic drift. Influenza vaccines are updated annually to ensure that antibody-mediated immunity is stimulated to the relevant predicted strains of the H3N2 and H1N1 subtypes of influenza A, and influenza B.

Haemagglutination inhibition assays are the current industry standard for measuring antibody responses to influenza vaccination as a proxy for vaccine efficacy. There is significant literature reporting a decline in antibody titres with aging summarized in a recent metanalysis of these studies [9]. However, many of these studies have not defined the health status of study participants, their vaccination status, or the setting in which they live. Studies of the antibody response to influenza vaccination over multiple influenza seasons comparing healthy young adults to relatively healthy adults (probably representing "usual aging") have shown no difference between these two groups [10, 11]. These results suggest that aging alone does not affect the antibody response to influenza vaccination as measured in hemagglutination inhibition assays, and thus do not explain the differences in vaccine efficacy between young and older adults. Furthermore, even though the antibody response to vaccination might be predicted to decrease with repeated vaccination in older adults, annual repeated vaccination, in fact, improves protection against influenza [12–14]. Another postulate for the differences in vaccine-mediated protection in young and older adults has been that the duration of the antibody response to influenza vaccination may be shortened in older persons and not provide protection through the influenza season. However, a recent review found no evidence in the published literature of a premature decline in antibody titres during the influenza season in community-dwelling older adults [15].

There has been a paradigm shift in understanding the limitations of antibody titres as a sole measure of influenza efficacy [16]. As a correlate of protection against influenza, our studies have shown that serum antibody titres are similar and do not distinguish between older adults who will go on to develop influenza illness from those who do not [17]; and (McElhaney, submitted for publication). This is not to say that antibodies are not an important defense mechanism, but emphasizes the point that both humoral and cell-mediated immunity are important for clinical protection in older adults [18]. Thus, the evaluation of antibody titres alone as a surrogate of vaccine efficacy may fail to correlate with estimates of vaccine effectiveness from epidemiologic studies.

2.1.3 T-Cell Responses to Influenza are Conserved Across Different Strains

In contrast to B-cells that mount a subtype and strain-specific response, the antigenic determinants of the T-cell response are more conserved across the different strains of influenza. Thus, T-cell recognition and the response to influenza does not

degrade with antigenic drift [19–21]. Internal peptide sequences of hemagglutinin and neuraminidase are similar within the subtypes of influenza A (e.g., A/H3N2 vs. A/H1N1). Internal viral proteins (matrix and nucleoproteins) are conserved within the types of influenza (e.g., influenza A vs. B) [22]. Thus, peptides derived from surface glycoproteins and internal viral proteins stimulate helper T-cell and CTL responses that are cross-reactive within the strains of influenza A or influenza B. In other words, antibody responses are relatively strain-specific, while T-cell responses are cross-reactive across strains within influenza A or B.

Previous studies have shown that exposure of the entire respiratory tract to live influenza virus is the most effective method of inducing cross-reactive T-cell responses to influenza virus infections [23]. A direct comparison between different routes of infection showed that protection correlated with the size of the virus-specific CTL (CD8+) response in the lungs and associated lymphoid organs. Although self-renewing populations of virus-specific CD8 T-cells are maintained in the lymphoid organs for many years after influenza and other respiratory virus infections, protective cellular immunity is short-lived and disappears within about 6 months [23]. However, this CTL memory response can be recalled by vaccination with split-virus influenza vaccines in older adults especially when the vaccine has been recently exposed to natural infection with influenza (McElhaney et al., submitted for publication).

2.1.4 Effective Stimulation of Helper T-cells and CTL

Virus is taken up and processed by antigen-presenting cells such as macrophages and dendritic cells, and the resulting peptides are presented with the major histocompatability complex to activate T-cells [24]. Helper T-cells (T_h) recognize antigens presented by the major histocompatibility complex Class II (MHC II); MHC II is expressed almost exclusively on antigen-presenting cells, B-cells and T-cells [25]. In contrast, CTLs recognize viral peptides in combination with MHC I; MHC I is expressed on most cells in the body [26]. Structural viral proteins and both live and inactivated viruses are phagocytosed by macrophages and dendritic cells. The virus is processed within the antigen-presenting cell and presented in combination with MHC II to helper T-cells [27]. In contrast, viral peptides presented in combination with MHC I, are generally the products of viral replication within the antigen-presenting cell, although antigen cross-presentation in dendritic cells does occur (discussed below). Thus, the form of the viral antigen, and the interaction with a specific MHC and its cellular location independently determine T_h and CTL responses to vaccination [28, 29].

Antigen cross-presentation is the process by which antigens including killed virus or viral proteins are taken up by the dendritic cells, undergo proteasomal degradation, and are processed for presentation on MHC I. Because killed virus (contained current parenteral influenza vaccines) is effectively presented on MHC II and not MHC I, T_h and B-cells are stimulated to produce good antibody responses, but only weak CTL responses are seen in adults; this CTL response

that is not seen in influenza-naïve individuals, results from restimulation of a previously primed response to influenza through natural infection [28–31]. This process is postulated to be the mechanism by which inactivated viruses including split-virus influenza vaccines can stimulate CTLs in populations primed by a previous influenza infection [30]. The relevance of antigen cross-presentation to new vaccine development is that Toll-like receptor (TLR) ligands [32], virosomes [33], virus-like particles [34], and potentially adjuvants [35] can be used to activate APC and enhance expression of MHC I-viral peptide complexes and improve the poor CTL responses elicited by the current killed virus vaccines in older adults. Boosting T-cell responses is an important priority for vaccine development, in general, due to broader protection against serologically distinct strains of virus [23, 36, 37]. Because immunosenescence alters several aspects of cell-mediated immune function, vaccine design can include independent strategies for effectively stimulating T_h and CTL responses.

2.1.5 Effect of T-helper Cell Function on the Response to Influenza

The T_h -mediated immune response to influenza virus plays a key role in the generation of both humoral and CTL responses to influenza vaccination. Previously, T_h1 and T_h2 were defined by their cytokine products such that the T_h1 cytokine, IFN-γ, down-regulated T_h2, and IL-10 downregulated T_h1 [38–40]. While this paradigm is generally applicable in the mouse model, recent studies have questioned the validity of the T_h1/ T_h2 paradigm in humans, and the contributions of regulatory T-cells (Treg or T_h3) and T_h17 subsets to cytokine regulation are only beginning to be understood [41]. Under a revised model, naïve CD4+ helper T-cells are stimulated by IL-12 to produce IFN-γ (i.e. become T_h1); IL-4 stimulates T_h2 to produce IL-4, IL-5, IL-13; and IL-1, IL-6 and IL-23 stimulate T_h17 to produce IL-17, IL-22 and IL-26. These T_h subsets have counter-regulatory interactions between each other [42]. Our data showed that the IFN-γ:IL-10 ratio correlates with risk for influenza illness [17] but characteristics of the vaccine recipient and PBMC culture conditions may alter this relationship [43–46]. The apparent down-regulation of IFN-γ by IL-10 may be T_h3-mediated rather than a shift from a T_h1 to a T_h2 response [47], and the interaction with T_h17 has not been studied at all.

T_h17 appear to have developed as part of the adaptive immune response to combat extracellular pathogens not covered by T_h1 or T_h2 immunity based on studies in mice [48]. Studies in human PBMC sharply contrast with the results in mouse models. T_h17 promotes the recruitment of IFN-γ producing T-cells and as such, is regulated by the tissue level of IFN-γ [49]. Recent studies in human PBMC have shown that T_h17 can simultaneously produce IL-17 and IFN-γ suggesting that the two cytokines may work synergistically in the adaptive immune response[50]. Given the centrality of the T_h17 subset in immune regulation, T_h17 may have an important role in determining the cytokine response to influenza and responses to influenza vaccination.

2.1.6 Potential Cytokine-associated Correlates of Protection Against Influenza

Aging leads to a reduction in IL-2 synthesis [51, 52], an increase in IL-4 production [53], dysregulation of T_h1 and T_h2 cytokine responses and a decline in the CTL responses to influenza [54]. However, a recent review of the application of the T_h1/ T_h2 paradigm in older adults highlights the discrepancies of results across a number of studies in older people [47]. In light of an evolving understanding of interaction of multiple T-cell subsets in humans, the response to influenza and influenza vaccination may be more complicated than predicted by these earlier studies. A reduction in the ratio of IFN-γ to IL-10 levels in response to ex vivo challenge of PBMC with live influenza virus, is associated with increased risk for influenza illness [17]. However, the source of IL-10 may be from multiple different T-cell subsets in these cultures including T_h3/Treg. Also, absolute cytokine levels are less likely to predict risk for influenza illness suggesting that it is the regulation of the different T-cell subsets that determines the response to influenza and clinical protection from illness. It may be the balance and regulation of T_h1/ T_h2/ T_h3/ T_h17 responses that is important for a protective response to vaccination in older adults and recovery from influenza illness [55–57].

Recently, it has been shown in mice that with aging, antigen-presenting cells including monocyte/macrophages and dendritic cells produce lower levels of proinflammatory cytokines in response to ligation of Toll-like receptors [58]. The addition of these cytokines (IL-1, IL-6 and TNF-α) to spleen cells can reverse these age-related defects in T helper type 1 cytokine production [59]. The paradox is that IL-6 levels increase with age, chronic disease and stressors of the immune system and contribute to a proinflammatory state with increased production of IL-6 [60] and thus should stimulate rather than suppress T_h1. These results reflect conflicting postulates as to the determinants of influenza risk in older people based on cytokine levels. The recent identification of T_h17 cells and their regulation through TGF-β and IL-6 production and T_h3/Treg [61, 62], may shed further light on differences in cytokine regulation, susceptibility and health outcomes, and the relationship with acute illnesses versus chronic diseases.

2.1.7 Potential CTL-associated Correlates of Protection Against Influenza in Older Adults

Human studies have shown that CTL activity is important for recovery from influenza infection even in the absence of protective antibodies to the infecting virus strain [63]. CTLs combat influenza viral infections by recognizing and destroying virus-infected host cells that become the factories for viral replication. Infected cells expressing on their surfaces the MHC I-viral peptide complex are recognized by and activate virus-specific CTL [26]. Two mechanisms by which CTL activation leads to lysis of virus-infected cells include perforin- or granule-mediated killing [64–66], and fas-mediated killing [67, 68]. Granule-mediated killing is particularly

important for the control of respiratory viral infections although fas-mediated killing may provide an alternative but less specific mechanism [69].

A direct comparison showed that protection correlates with the virus-specific CTL (CD8+) response in the lungs and associated lymphoid organs. Although self-renewing populations of virus-specific CD8 T-cells are maintained for many years after influenza infection, protective cellular immunity is short-lived and disappears within 6 months [23, 36, 70]. Even though current inactivated influenza vaccines stimulate a CTL response in older and even chronically ill older adults [71], this response is diminished compared to young adults [72–74] and is not as robust as the response to natural infection [75]. As well, the degree of cross-reactivity of CTL responses for different subtypes of influenza may decrease in chronically ill compared to healthy older adults [71, 72].

Virus-specific killing is mediated by granzymes contained in granules within CTL. Granules migrate to the "immune synapse" between the activated CTL and the virus-infected target cell, are transported across the cell membrane into the cytoplasm of the target cell, and are involved in an enzymatic cascade that leads to apoptotic cell death [76]. Granzyme B (GrzB) is a key element of the T-cell response to influenza in the lung [77–79]. An assay of GrzB activity in lysates of influenza virus-stimulated PBMC correlates with cytolytic activity by standard ^{51}Cr-release assays [80, 81] but has the advantage of being a more sensitive measure of cytolytic activity that is detectable in ex vivo virus-activated PBMC. Ex vivo levels of GrzB in lysates of influenza-stimulated PBMC correlate with risk for influenza illness in older adults [17]. Other ex vivo studies have shown no difference in influenza-specific CTL frequencies in older compared to young adults [82]. Taken together, these studies suggest that in influenza susceptible older persons, there is defect in the amount of GrzB produced on a per CTL basis.

2.1.8 Interaction of Antibody and Cell-mediated Immune Response to Influenza Vaccination

Current killed virus vaccines effectively stimulate T helper cells and vaccination of healthy older people increases IL-2 to levels comparable to that of young adults [83–85]. Other studies have reported heterogeneous cytokine responses to influenza vaccination in healthy older people that are related to characteristics of the vaccine recipient and the vaccine [43–45]. The in vitro proliferative response to influenza vaccine is associated with protection from influenza illness in ambulatory older adults [18]while antibody titres, tested in different settings, have not been consistently associated with risk for influenza illness in older people [17, 86]. In institutionalized older adults, IL-2 and IFN-γ responses to influenza are significantly associated with the level of independence in activities of daily living but do not predict protection against influenza illness [46]. Current killed virus vaccines have been shown to decrease the IL-10 (T_h3/Treg) response to influenza for an overall increase in the T_h1 (IFN-γ) relative to the T_h3 response [87], but the short duration of the response is not effectively re-stimulated with a booster vaccination [88]. Further

the ratio of IFN-γ:IL-10 has been shown to predict a protective response to influenza vaccination [17].

Killed virus vaccines stimulate a weak cytotoxic T-cell response and have limited efficacy in older adults. New developments in vaccine technology that improve the regulation of T_h cytokines and potentially increase the CTL response look promising**.** Live-attenuated intranasal vaccines were developed to provide more effective stimulation of CTL. However, these vaccines have shown minimal additional benefit for preventing influenza in older adults when combined with the standard inactivated parenteral vaccines, although some improvements in symptoms [89] and immunogenicity [90] have been reported. Thus, the currently available killed virus vaccine given by injection is still the recommended vaccine for those aged 50 years and older.

2.1.9 The Effect of Replicative Senescence on Immune Function and Responses to Vaccination

With aging and the multiple immune responses that have been stimulated throughout one's lifetime, there is a gradual shift from predominantly naïve T-cells to increasing proportions of memory T-cells. Thymic involution and the loss of naïve T-cells with aging may thus exhaust the capacity to respond to new antigens. Recent studies have further delineated central and effector memory helper T-cells, and have shown that healthy older adults have T_h responses to influenza vaccination similar to young adults. However, an age-related decline in IL-7 levels corresponds to failure to maintain or expand the effector memory helper T-cell response to influenza [91]. The importance of memory T-cells in recalling the response to the many cross-reactive influenza epitopes may be a key element of both T_h and CTL responses to split-virus vaccines.

Features of 'successful aging' have been associated with well-preserved immune function while poor survival is predicted by high CTL counts, low helper T-cell counts, low numbers of B-cells and poor responses by T-cells to polyclonal stimulation [92, 93]. The phenomenon of replicative senescence has been associated with these changes and relates to the finite number of doublings (25–30 cycles) after which cell cycle arrest occurs [94]. In CTLs, this growth arrest is associated with increased production of several proinflammatory cytokines, resistance to apoptosis [95], and loss of the costimulatory molecule, CD28, required for optimal stimulation of CTLs [96, 97]. CTL that do not express CD28 (CD28- CTL) have little or no cytolytic activity [98] and an increased proportion of CD28- CTLs is associated with a decline in antibody responses to influenza vaccination [99, 100] and a reduction influenza-specific memory CTL [101]. These changes have been associated with chronic cytomegalovirus infection driving the T-cell response to terminal differentiation and expressing this senescent phenotype [102, 103]. However, it remains uncertain the extent to which this change may affect the T_h and CTL responses to influenza and influenza vaccination.

2.1.10 Summary

Influenza is a serious illness in older adults and largely accounts for rising hospitalization and death rates from acute cardiac and respiratory illnesses in older people despite widespread influenza vaccination programs. While current vaccines are cost-saving, new influenza vaccines will be needed to avoid the anticipated crisis in health care related to the aging of the population. Recent studies suggest that there is a significant opportunity to exploit the reserve capacity of T-cells to respond to influenza antigens through enhanced antigen presentation, appropriate costimulation, and regulation of cytokine responses. Targeting identified immunologic mediators that modulate influenza risk in older people, and screening candidate vaccines for clinical trials using appropriate correlates of protection in this population, is critical to development of more effective influenza vaccines for an aging population. Since the early phases of vaccine development often rely on antibody titres as a surrogate of protection, this measure may fail to detect a more robust T-cell response and thus, a more effective vaccine for the 65 and older population.

2.2 New Vaccines for Herpes Zoster

2.2.1 Risk for Herpes Zoster and Aging

Herpes zoster (or Shingles) is a painful blistering rash resulting from the reactivation of latent varicella-zoster virus (VZV), the agent that causes of chickenpox. Prior to routine vaccination for VZV, approximately 90% of people in the USA were infected with this virus and the chance of developing shingles during one's lifetime was 25–30%. The risk of developing shingles dramatically increases with age.

Older persons bear the greatest burden of illness related to shingles, the clinical condition that results from reactivation of latent varicella-zoster virus (VZV). Each year between 600, 000 and 1 million Americans develop shingles and the risk dramatically increases with age—50% of persons over age 85 will suffer from disabling post-herpetic neuralgia (PHN) as a complication of shingles. Despite extensive epidemiologic studies of risk, little is known about the immunologic determinants of risk for shingles. The age-related decline in T-cell function is well documented but there is only limited data on how T-cell responses to VZV change with aging. Further, as shingles is exclusively a human herpes virus, animal models are very limited and may not be helpful in identifying the mechanism by which aging precipitates shingles. Particularly due to the aging of the "Baby Boomers" the age 65 and older population will grow to represent 20% of the US population over the next three decades. Identifying the immunologic changes in the response to VZV that occur with aging, is the first step in a mechanistic approach to targeting vaccines for this important human disease.

2.2.2 Shingles is an Important and Disabling Disease in Older Adults

Varicella-zoster virus presents as chickenpox in childhood and becomes a latent infection in the dorsal root ganglion of the spinal cord. The increased risk of shingles with age has been well-documented with an annual incidence of 14/1000 in those age 75 years and older leads and an astonishing prevalence of up to 50% of those over age 85 years old [104–106]. Reactivation causes a painful dermatomal rash called shingles that is often followed by PHN, a chronic pain syndrome associated with significant disability in older people. The incidence of PHN is almost negligible before age 50, but 21% of patients older than 60 years and 29% beyond age 70 become affected following an attack of shingles. This contrasts with shingles in children where the rash generally follows a mild case of chicken pox and is of little clinical significance [107]. Antiviral therapy is available but older people often do not present within the 48–72 hour window of onset of the rash necessary for initiation of effective treatment. In addition, 20% of older people who receive therapy within the therapeutic window still experience pain six months after the onset of the rash [108]. Particularly given the number of people who do not seek or receive timely and effective treatment of shingles, the prevalence of disability related to PHN is a major public health concern.

There is a significant literature on the impact of shingles on the quality of life in older people and on various therapeutic strategies for the management of PHN, the review of which is beyond the scope of this review that focuses on the prevention of shingles. However, the importance of perceived quality of life and psychological conditions that have been identified from epidemiologic studies as risk factors for the development of shingles, are relevant due to their potential impact on immune function. A recent review of these studies suggests that in addition to age, poor self-perceived health, psychological stress and/or lack of social support and mechanical trauma may lead to loss of cell-mediated immunity to VZV and increased risk of shingles [109].

2.2.3 Studies of the Link Between Risk for Shingles and Immunosenescence are Limited

Because VZV is exclusively a human Herpes virus [110], there are limited animal models that study only some aspects of VZV infection and reactivation [111]. From the studies to date, it appears that with the resolution of chickenpox, VZV-specific CTL access the dorsal root ganglion where VZV lives, to keep viral replication in check [111, 112]. At some point the virus escapes to replicate in nerves and skin to cause a very painful condition that continues even after the rash resolves. Whether or not reactivation of VZV is due to a general decline in CTL-mediated immunity or due to changes in VZV-specific CTL is unknown. These findings have not been studied as a potential mechanism for reactivation of VZV in older people. Further, the mechanism that keeps virus restricted to the dorsal root ganglion is unknown and it may be postulated that reactivation of VZV in older people is due to an increased number

of CD8+CD28- VZV-specific CTL that produce IFN-γ but do not contain cytolytic mediators such as Grz B. This hypothesis would be consistent with the extensive literature on the age-associated loss of CD28 expression, telomerase activity and telomere length affecting both CD4+ and CD8+ T-cells that is also associated with repeated antigenic stimulation (Reviewed in [94]).

Reactivation of VZV is associated with marked inflammation of the dorsal root ganglion leading to nerve cell damage and the pain associated with PHN that often precedes the onset of the dermatomal rash. Inflammatory cytokines produced by a stressful event and in the early stages of VZV reactivation may further suppress CTL function. With aging, there is a loss of ability to downregulate the inflammatory response and probably leads to excess nerve damage in the dorsal root ganglion and increased pain that persists as PHN for greater than one year in more than 50% of adults age 70 years and older who experience PHN. Even appropriate antiviral treatment initiated within 72 hours of onset of the rash fails to prevent this complication. Clearly, re-establishing the normal immune response to this virus requires the stimulation of VZV-specific CTL and regulation of the appropriate cytokine response to suppress viral replication without causing inflammation.

2.2.4 The Loss of the Costimulatory Molecule, CD28, Affects Immune Function

Age-related changes in T-cell function have been associated with terminal differentiation of memory T cell and replicative senescence (previously discussed in Section 2.1.9 and Reference [93, 94, 96, 98]) and an overall age-related decline in VZV-specific T cells. These changes lead to an increased risk of reactivation of VZV and the development and probably severity of PHN in older adults. Although suppression of VZV is unlikely to drive the general process of terminal differentiation of T cells as is the case with CMV, the loss of CD28 on VZV-specific T cells and co-stimulatory function, is a likely contributor to the risk for shingles and PHN. Effective vaccines against shingles may therefore need to stimulate T-cell subsets that express CD28 costimulatory molecules [113] and respond to novel strategies for antigen presentation.

2.2.5 The Development of a Shingles Vaccine

A large randomized double-blind placebo-controlled of a shingles vaccine enrolling over 38, 000 subjects showed in the vaccinated compared to the placebo group, a 61.1% reduction in burden of illness, a 51.3% reduction in shingles cases, and 66.5% reduction in those shingles cases complicated by post-herpetic neuralgia [114]. Futhermore, there was a reduction in the overall burden of illness in vaccinated subjects showing statistical significance for the primary endpoint in the trial. The vaccine strain of VZV is a previously attenuated live virus (Oka strain) that is predicted to stimulate humoral, T_h and CTL responses. Importantly for this disease, antibody titers do not predict protection from reactivating the virus to cause shingles. The postulated mechanism of protection is stimulation of the VZV-specific T-cell response to vaccination and meas-

ured by the IFN-γ enzyme-linked spot (ELISpot) assay [115, 116]. Given that this a live attenuated vaccine, safety testing included isolation of virus from all shingles cases following vaccination. Specimens collected from skin lesions in shingles cases in the post-vaccination period showed wild-type strains; the vaccine Oka zoster strain was not identified in any of the isolated specimens suggesting that this attenuated virus can be safely and effectively used to stimulate the senescent immune response. Since this clinical trial had relatively few exclusion criteria, the results of this clinical trial should be applicable to most adults age 60 or older who are not immunocompromised due to underlying diseases or medications.

2.2.6 Summary

Shingles is a major debilitating disease in the older adult population. Both age-related and age-associated changes in the cell-mediated immune response to VZV are clearly associated with increased risk of reactivating the virus to cause shingles and persist as PHN. The fact that antiviral therapy has limited effectiveness in the treatment of zoster in older adults points to the need for strategies to prevent the disease and it disabling complications. However, the development of a vaccine against Herpes zoster depended on a very large clinical trial to determine vaccine efficacy based on clinical outcomes. In the absence of reliable immunologic markers of vaccine efficacy, there was significant risk that the vaccine would fail to show an improvement. If the vaccine had failed in this trial, there may have been limited interest from industry in moving forward with alternate plans to develop an improved vaccine. This again points to the need for more reliable surrogates of vaccine efficacy to test new vaccines in the early phases of clinical development and select for subsequent clinical trials, the vaccines that are most likely to improve outcomes in the 65 and older population.

2.3 Implications of Effective Vaccines Against Respiratory Syncytial Virus

2.3.1 Respiratory Syncytial Virus Causes Serious Respiratorty Illness in Older Adults

RSV is a commonly circulating virus during the winter months and accounts for 2–5% of pneumonias in community-dwelling older adults (reviewed in [117]). The importance of this respiratory illness, particularly in older adults is increasingly recognized; it was recently reported that RSV causes 10,000 excess deaths in the United States and is second only to the A/H3N2 strains of influenza as a cause of death due to viral respiratory illness in the age 65 and older population [2, 118]. Although the virus is genetically stable over time (in contrast to influenza), repeat infections throughout adult life are common suggesting that immunity to this virus wanes over time. Those older adults with increased risk for severe disease are those with congestive heart failure and chronic lung disease, the severely immunocompro-

mised, and those living in long-term care facilities [119]. Estimates of RSV disease in this setting range from 5–10% of residents per year with pneumonia and death in 10–20% and 2–5% of cases, respectively. As with influenza illness in older adults, RSV results in prolonged lengths of hospital stay, significant disability and loss of independence in basic activities of daily living, and the need for a higher level of care at hospital discharge [120].

Studies of the immune response to RSV have shown that high levels of serum and/or nasal antibodies have been correlated with relative resistance to experimental challenge. Similarly, low serum and nasal antibody levels are risk factors for infection and disease severity in older adults but this is not an age-specific change [121, 122]. More importantly, older adults have a greater rise in antibody titres postinfection than do their younger counterparts [122]. Since the RSV virus does not undergo antigenic drift over time, one would predict better protection against recurrent RSV illness in older adults but instead there is a relative increased risk of RSV infection with aging. This observation may be explained by an age-related shift from a T_h1 to a T_h2 response to RSV, which has been shown to cause significant pathology in people. In the aged mouse model, diminished CD8+ CTL responses associated with decreased IFN-γ (T_h1) and increased IL-4 (T_h2), and higher RSV titres in lungs [123]. However, recent studies in human PBMC show no age-related changes in cytokine levels produced in response to RSV although the regulatory balance between inflammatory (IFN-γ and antiinflammatory (IL-10) cytokine levels may be altered [124].

2.3.2 The Development of a Respiratory Syncytial Virus (RSV) Vaccine

RSV circulates through much of the winter and often cocirculates with influenza during the mid-winter months. This presents a diagnostic challenge to clinicians as the symptoms of RSV illness completing overlap with those related to influenza illness [125]. Thus, treatment approaches would be particularly problematic as a strategy for limiting the complications of RSV and none are currently available for use in adults. The development of a vaccine against RSV has proven to be a significant challenge, perhaps due to the reliability of antibody titers as correlate protection in these trials. Deaths were observed in RSV-naïve children in whom RSV infection restimulated the immune response to vaccination and resulted in a significant inflammatory response to RSV infection. The challenge to developing an RSV vaccine for older adults is that RSV illness in older adults has not been well-studied, the virus circulates over a larger proportion of the winter months compared to influenza, and the symptomatology overlaps with influenza.

In summary, older adults experience significant complications of RSV illness but these complications are difficult to distinguish those related to influenza. Based on attack rates and impact of hospitalization in older adults, RSV is likely to cause significant disability in older adults. Immunologic correlates of clinical protection are not available and this presents a significant challenge to the development of the much needed vaccines against RSV for older adults.

3 Immune Senescence: Stimulating a Naïve Response

3.1 Pandemic Influenza Vaccines for Older Adults

The threat of pandemic influenza has increased with the direct transmission of highly pathogenic avian H5N1 viruses to humans and many countries are in the process of or have completed plans to manage an anticipated influenza pandemic. While animals have transmitted H5N1 influenza to people in close contact with livestock, additional mutations or reassortment events will be required for widespread human-to-human transmission. Current research is focused on predicting the strains that are likely to evolve so that new influenza vaccines can be developed to protect against these new strains. The development of effective pandemic influenza vaccines is likely but continued reliance on killed virus or subunit vaccine technology will leave older adults at significantly higher risk of illness, disability and death in the event of an influenza pandemic.

Targeting improvements in T-cell responses and thus protection against a number of strains will be particularly helpful for stimulating the senescent immune system against both seasonal and pandemic strains. In the case of H5N1, vaccines will not only have to stimulate an antibody response to the new vaccine strain but will also have to prime the T-cell response to influenza peptides derived from H5; age-related changes in naïve T-cells would result in decreased production of IL-2 and hence, the proliferative response to the vaccine in both B- and T-cells. This has implications for both prepandemic and pandemic vaccines. Pre-pandemic vaccines if formulated to more potently stimulate T-cells could offer cross-protective immunity and would enhance the production of strain-specific antibodies against the pandemic strain. Although this strategy may offer enhanced protection in older adults, prepandemic and pandemic vaccines will need to be tested for their ability to stimulate adequate antibody responses and cross-protective cell-mediated immunity. In the absence of improvements in the current split-virus vaccine technology, an influenza pandemic could have a significant impact on older people with overwhelming consequences for the health care system.

3.2 Other Viruses

As individuals age, infectious diseases cause increasing morbidity and mortality. This is especially evident when older adults contract newly emerging diseases such as severe acute respiratory syndrome (SARS), which killed 50% of infected individuals over the age of 50 [126]. The rapid human-to-human transmission of SARS exposed the entire age spectrum to a novel virus and highlighted the changes in the immune system that lead to increased morbidity and mortality rates with aging. Fortunately the outbreak was controlled without a vaccine and before it could reach epidemic proportions. Older adults may also be naïve to viruses such as West Nile Virus

(WNV) and Human Immunodeficiency Virus (HIV) and appear to be at increased risk of serious complications. When these viruses are contracted by an aged host, the senescent immune system may produce a less effective response compared to young adults. Evidence for this decline is from epidemiologic studies showing much higher mortality rates in older compared to young adults with WNV [127].

HIV prevalence is increasing and with aging of the population, HIV-infected patients age 50 years and older now represent10–13% of the HIV-infected population in the United States [128]. Both HIV and aging have been associated with the development of replicative senescence of T-lymphocytes and increased risk of infection [129]. Replicative senescence results from chronic stimulation of the immune system by the HIV virus and is associated with telomere shortening and loss of CD28 expression on CD8 T-cells [130]. These changes will need to be considered in the development of new therapies to improve the immune response to viral infections and vaccination [131].

4 Immune Senescence: Vaccines Against Bacterial Pathogens

4.1 Pneumococcal Vaccination

Streptococcus pneumoniae is an important cause of morbidity and mortality as a leading cause of community acquired infections including bacterial pneumonia, meningitis, and bacteremia. Amongst the highest risk groups and who bear the greatest burden of disease in the developed world, are adults age 65 years and older. The current 23-valent vaccine containing pneumococcal capsular polysaccharide (PPS) is cost-effective in this population [132, 133], but its efficacy may be limited by age-associated changes in the immune response to these vaccines. Although there is no age-related decline in the antibody response to pneumococal vaccination when healthy young and older adults are compared, consistent antibody responses to all 23-serotypes contained in vaccine may not be achieved in older adults [134]. In addition, opsonophagocytic activity, the major effector mechanism for clearing pneumococcus appears to decline with aging [135]. Further, there is a significant decline in antibody titres to PPS at six years following pneumococcal vaccination [136]. Repeat vaccination at least every 6 years in older adults may be needed to maintain protection against pneumococcal disease and can be safely administered in older adults.

Current vaccines containing PPS stimulate antibody production through a T-independent type 2 (TI-2) response (one not requiring T-cell help and lacking memory) [137]. Given that it is primarily cell-mediated, rather than humoral immunity that declines with the normal aging process, the efficacy of pneumococcal vaccines in older adults may also depend on how the cell-mediated immune response to the whole pneumococcus is stimulated. A Finnish study of older adults, showed that serotype-related differences in the serum antibody response following vaccination with 23-valent capsular polysaccharide vaccine, suggesting that some serotypes are weak immunogens in older adults [138]. Protein-conjugated PPS vaccines have

been developed to facilitate T-cell cooperation [139] and are effective in children. Fewer serotypes are represented in these vaccines and their benefit over traditional polysaccharide vaccines, have not been demonstrated in older adults.

Cytokine responses to pneumococcal antigens have been shown to regulate responses to protein and polysaccharide-specific antibody responses [140] and may be important in the pathogenesis of pneumococcal diseases [141]. Cytokine responses to the whole pneumococcus could explain changes in the virulence of different serotypes in older adults [142] and in comparison to cytokine responses in younger adults [143]. TNF-α, a macrophage product, is associated with an initial inflammatory response to pneumococcal invasion [139, 143] and has been found to be important in the development of antibodies to pneumococcal surface proteins [140, 145]. T_h2 cytokines including IL-4, although classically involved in stimulating B-cells to produce antigen-specific antibodies, may downregulate antibody responses to pneumococcus due to inhibitory effects on antigen-presenting cells [140]. IL-10, a product of both macrophages and T_h3 lymphocytes, is an antiinflammatory cytokine that also skews the in vivo immune response toward a T_h2 (humoral) by inhibiting a T_h1 (cell-mediated) response [146]. Increased IL-10 levels in animal models have been associated with increased risk for pneumococcal infection [142]. IL-12, a product of both phagocytic and antigen presenting cells, is a potent proinflammatory cytokine with a key role in resistance to bacterial infections. IL-12 upregulates the T_h1-mediated responses (IFN-γ) which recruits neutrophils to the lungs and thus has a protective role in the response to pneumococcus [147, 148]. Dysregulation of T_h-mediated cytokine responses with aging may thus contribute to the increased risk for pneumococcal infection in older adults. A greater understanding of the interactions between cytokine and antibody responses to pneumococcus and the immunologic determinants of risk for pneumococcal diseases are needed if improved vaccines are to be developed in older adults.

5 Immune Senescence: Altering Responses to Endogenous Proteins

5.1 Vaccines for Alzheimer's Disease

Alzheimer's Disease (AD) is caused by the deposition of β-amyloid protein (Aβ) in the brain with toxic effects leading to neuronal cell death, amyloid plaque formation and the development of neurofibrillary tangles. Based upon studies in mice, a vaccine containing the $A\beta_{1\text{-}42}$ peptide was shown to stimulate antibody production and improved cognitive function in mouse models of AD. This vaccine was advanced to a Phase II clinical trial based on the demonstration of no significant adverse effects of vaccination in a Phase I trial that included 200 subjects. The Phase II trial was halted due to the development of aseptic meningoencephilitis in 6% of the 300 vaccinated subjects. Analysis of the antibody response to Aβ showed a trend toward

cognitive enhancement in the "responders" to vaccination (antibody titre to $A\beta_{1\text{-}42}$ ≥ 1:2200) but 22% of "responders" compared to 2% of "nonresponders" developed aseptic meningoencephilitis [149]. These results suggested that adverse effects of the vaccine were related to the immune response to the vaccine rather than toxicity of the $A\beta_{1\text{-}42}$. Postmortem studies of the meningoencephilitis cases showed substantial clearing of Aβ from the brain but with marked T-cell infiltration in brain tissue.

The postulated age-related defect that leads to the pathology of AD related to APC uptake of Aβ and stimulation of T_h1 cytokines, is an inflammatory response to Aβ. This defect is associated with inefficient phagocytosis of Aβ and the production of inflammatory cytokines (IL-1β, TNF-α) and chemokines, and nitric oxide leading to complement activation and T-cell apoptosis [150]. The immune mechanism being targeted by the vaccine was T-cell-dependent antibody production against Aβ to form Aβ-antibody complexes for more efficient clearing of the Aβ. Earlier studies had shown that $A\beta_{1\text{-}42}$ effectively stimulated a proliferative response in human peripheral blood mononuclear cells. This response was increased in older compared to young adult subjects and a further significant increase was observed in older adult subjects with AD [151]. It had been shown that $A\beta_{1\text{-}15}$ was responsible for B-cell stimulation and the production of antibodies to Aβ, and $A\beta_{15\text{-}42}$ most effectively stimulated T-cell proliferation and the production of both T_h1 (IFN-γ) and T_h2 (IL-13) cytokines. Because a T_h1 (vs. T_h2) response in the AD mouse model was associated with more effective clearance of Aβ in the mouse model, QS21 adjuvant was added to the Aβ vaccine used in human trials to stimulate a Th1 response to the vaccine. It is postulated that the adjuvanted vaccine activated Aβ-specific memory T-cells that migrated to the sites of Aβ deposition in the brain and produced Th1 cytokines. Although the antibody response appeared to effectively clear Aβ, the inflammatory cytokine response of the T-cell infiltrate lead to meningoencephalitis. Since the $A\beta_{15\text{-}42}$ stimulated both Th1 and Th2 cytokines, it appears that the addition of the QS21 adjuvant may have been responsible for the serious adverse effects of the Aβ vaccine [150]. While efforts continue to develop immunologic-based therapies for AD, the results of this clinical trial will have significant consequences for future vaccine development. Lessons learned from the Alzheimer vaccine trial suggest that targeting the aged immune system through vaccination to produce a more effective response may be a "double-edged vaccine"[152].

6 Summary

Age-related changes in the immune system have been associated with increased risk for infectious diseases. These are largely attributed age-related changes in T-cell-mediated immunity and defects in defense mechanisms mediated by Th1 (IFN-γ) and CTL. This would suggest that more potent vaccines for older adults should stimulate Th1 and CTL to a particular pathogen. However, the underlying mechanism for defective immune responses in older people remains poorly understood including the potential negative impact of elevated levels of inflammatory cytokines (including IFN-γ). In

spite of our limited understanding, a number of available vaccines have been shown to cost-effective and even cost-saving in older adults. Future research to better understand the immunologic targets for the prevention or treatment of a variety of acute and chronic diseases will make a significant contributions to "adding life to years".

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Can Interventions to Influence Immunosenescence Suc

Interleukin -7 and Immunorejuvenation

Wayne A. Mitchell and Richard Aspinall

Contents

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Population ageing is one of humanity's greatest triumphs. It is also one of our greatest challenges. As we have entered the 21st century, global ageing will put increased economic and social demands on all countries. At the same time, older people provide a precious, often-ignored resource that makes an important contribution to the socioeconomic fabric of our lives. Population ageing raises some worrisome questions for policy-makers. Now that people are living longer, how can we improve the quality of life in old age?

'Advocacy on active ageing' from World Health Organization

W. A. Mitchell (✉) · R. Aspinall
Imperial College of London
Faculty of Investigative Sciences
Department of Immunology
369 Fulham Road
SW10 9NH, London
Tel: +44(0)208 746 5979
E-mail: w.mitchell@imperial.ac.uk

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With the global population experiencing increases in life expectancy, predictions suggest that nearly 50 % of the Western society will be composed of individuals over the age of 60 years by 2,050 (Steel and Maggi 1993). The challenge to us all is how to ensure that the prolonged life is free from illness and disease. It is well recognized that old age is associated with higher incidences of cancers, autoimmunity disorders and susceptibility to acute infections. This is further precipitated by the reduced ability to combat these illnesses and a decline in the responsiveness to the protective effects of vaccination. Taken together these factors indicate a major deleterious impact on the effectiveness of the functional immune system with advancing age. In this review we will examine the current scientific strategies and ideas for rejuvenating of the ageing immune system, with a particular interest in the potential role for interleukin 7, as we try to meet the challenges posed by the ageing population. The review will focus on four main areas. First, the impact of aging on the T-cell arm of the immune systems; second, the impact of cellular interactions on rejuvenation the age thymus; third, methods for generating functional T-cells ex vivo and fourth, adoptive transfer of T-cells as applied in the clinical setting.

1 Section I: Impact of Ageing on the Immune System

In order to better understand how the immune system can be rejuvenated we first need to appreciate the changes experienced by the immune system as we age. In particular, how the involution of the thymus alters the production and composition of the peripheral T-cell pool as a resulting from the reduced ability to generate of naïve T-cells necessary to orchestrate the immune response.

1.1 The Thymus and T-cell Component of the Immune System

The thymus is a primary lymphoid organ located in the anterior mediastinum and produces T-cells throughout life although the number of T-cells it produces declines with age (Hirokawa 1992; Makinodan and Kay 1980). Functionality of the thymus relies on, [1] an adequate supply of bone marrow derived precursor cells; [2] a number of extrinsic (endocrine) signals and [3] a thymic stroma that provides developing T-cells with a suitable microenvironment (Garcia-Suarez et al. 2003). Histologically the thymus is composed of two key components; [1] Thymic epithelial space in which thymopoiesis occurs and [2] Nonepithelial perivascular space (Haynes et al. 2000). The organ reaches a maximum size of approximately 25 cm^3 within the first 12 months of life (George and Ritter 1996). From this point thymopoietic thymic space has been observed to begin to atrophy shrinking in volume by 3 % per year until middle age and then by less than 1 % per year for the remaining years of life thereby reducing the capacity to develop

thymocytes (Steinmann 1986; Steinmann et al. 1985). At this rate it is estimated that total loss of thymic tissue will occur by 105 years of age. In a young healthy adult (less than 30 years old) there are approximately 2×10^{11} T-cells of which 1–2 % can be found within the blood, approximately 50 % of these cells are contained within the "antigen naïve" population. These T-cells have not interacted with their cognate antigen. Their activation requires a number of steps including recognition of the specific antigen presented in the appropriate MHC molecule in conjunction with the necessary costimulatory molecules by an antigen presenting cell. Age-related changes to the histological composition of the thymus culminating in a reduction in the number of naïve T-cell capable of responding to new antigenic assaults. These cells are required to provide a homeostatic balance between memory and naïve cells located within the T-cell pool. The resultant effect of thymic involution is that the composition of the T-cell pool is skewed toward memory T-cells (Fry and Mackall 2002a).

1.2 Generation of T-cells

Production of $\alpha\beta^+$ T-cells in the thymus is a progressive step-wise differential process, in which a small population of multipotential stem cells give rise to progeny populations. Stem cells migrating to the thymus are contained within the $CD4^-CD8^-$ double negative (DN) population, a population which has been further subdivided on the basis of expression of CD44 and CD25. Progress from the most immature stage, $CD44^+CD25^-$ (DN-1) requires the transient acquisition of CD25 so the cell first becomes $CD44^+CD25^+$ (DN-2) before becoming $CD44^-CD25^+$ (DN-3) and then the loss of CD25 when the population is $CD44^-CD25^-$ (DN-4; Godfrey et al. 1993, 1994; Wu et al. 1991). Cells within the DN-1 population are multipotential, whilst those at DN-2 have lost the capacity to form B cells, but can still produce either T-cells or dendritic cells (Shortman and Wu 1996; Wu et al. 1996). By the time the cells are within the DN-3 population they are committed to becoming T-cells and have undergone extensive rearrangement of the TCRβ chain genes (Capone et al. 1998). Expression of the TCRβ chain at the thymocyte surface requires a TCRα chain equivalent (Fehling and von Boehmer 1997; the preTCRα) and these cells then undergo expansion and differentiation so that they become $CD4^+CD8^+$ thymocytes. These immature thymocytes are the largest subpopulation in the thymus and are located in the densely packed cortical region of each thymic lobule. It is in the double positive stage when the TCRα chain undergoes rearrangement (Petrie et al. 1993) after which there is TCR αβ -dependant selection. Many of these double positive cells fail to mature further, but a small percentage develops into mature thymocytes expressing either CD4 or CD8 alone and is located in the medullary region of each thymic lobe. Only a fraction of these cells are exported to the periphery as naive or virgin T lymphocytes.

In a successful immune response, activation of these antigen naïve T-cells leads to their clonal expansion, the generation of effector cells and the subsequent reduction

in the amount and source of the antigen. This is followed by a period of cell death since the immune system no longer requires large numbers of T-cells bearing that specific receptor. However some cells with this antigenic specificity remain to become memory T-cells and subsequently enter the memory T-cell pool. Repeated exposure of the immune system to the same pathogen will be met by these memory T-cells and will lead to a response that is more rapid and of greater magnitude than the response following the initial exposure. This immunological memory provides the rational basis for protection by vaccination.

1.3 The Role of the Immune System

Since there are few completely sterile environments, each of us is confronted on a daily basis with different organisms, some of which could be pathogenic if we were not protected by our immune system. Our survival therefore depends upon the immune system recognizing and responding successfully to a broad range of potential pathogens. Provided these pathogens do not result in death, the immunological memory should increase, and analysis shows that this is indeed the case and that ageing is indeed associated with an increase in the number of memory T-cells.

1.4 Age-Related Effects on the Immune System

Theoretically then we should be able to cope with more infections as we get older; the immune system of a 90-year-old should be more experienced and therefore much better equipped to cope with infection than the immune system, of a 20-year-old. Unfortunately this does not seem to be the case, evidence from epidemiological, clinical and laboratory studies suggest an age related defect in the immune system. In reality, epidemiological evidence reveals a reduced ability to combat recurrent infection and that older individuals are often the first to be affected by new or emerging pathogens. For example, in the first outbreak of West Nile Virus in the USA in 1999 the median age of the 59 patients was 71 years, with 73 % of infected individuals aged 60 years or greater (Nash et al. 2001). In Israel, in 2000, all of the victims of West Nile Virus were more than 78 years of age (Berner et al. 2002).

Clinicians recognize that in addition to this susceptibility to new pathogens, older individuals often have difficulties in dealing with pathogens which they have previously overcome. Common problems include reactivation of herpes zoster virus (Schmader 2001) or the increased immune response to cytomegalovirus (Pawelec et al. 2004), as well as the problems associated with the yearly return of influenza and respiratory syncytial virus (RSV). For example in the USA from 1990 to 1999, influenza and RSV accounted for 51,203 and 17,358 deaths annually, respectively (Thompson et al. 2003). Vaccination trials also reveal problems with inducing protection in the elderly. Observation during a recent trial in which 45

healthy elderly (average age 74) and 37 healthy young controls (average age 28) were vaccinated with hepatitis B demonstrated that a protective titre was developed in all of the young individuals compared to only 42 % of the elderly cohort (Looney et al. 2001). A similar problem with vaccine cover occurs with influenza. Efficacy for influenza vaccine is between 70 % and 90 % in those under 65 but is reduced to 30–40 % in those over 65 (Hannoun et al. 2004).

Although several attempts have been made in the past to modify vaccines, either through alterations in their route of administration, or changes to their formulation by the inclusion of different adjuvants, the overall result has been a failure to improve the efficacy of vaccines in elderly individuals (Belshe et al. 2004; Looney et al. 2001). These trials would indicate that defects in the immune system rather than the deficiencies in the vaccine formulation are at the root of the problem.

Attempts to link these epidemiological and clinical results with laboratory studies has shown that T-cells from elderly individuals produce poorer proliferative responses in vitro to stimuli which are normally mitogenic for T-cell from younger individuals (Pawelec et al. 1997). Moreover phenotypic analysis of T lymphocytes from older individuals reveals that they have a different profile of cytokine gene expression (Bui et al. 1994) and there may be increased numbers of senescent T-cells than younger individuals (Effros et al. 2005). Like most somatic cells, T-cells have a limited replicative capacity and ageing is often accompanied by the increase in the number of T-cells present in the blood which have reached this replicative limit (Effros and Pawelec 1997). As we noted above, a successful immune response requires clonal expansion of antigen specific cells and the accumulation of T-cells without the capacity to divide can only lead to a dysfunctional immune response and failure to protect the individual.

1.5 Effects of Microenvironment on the Thymic Function: Young versus Old

Loss of thymic function associated with ageing significantly reduces thymic mass and impairs the ability to regenerate normal T-cell numbers after T-cell depletion. It is unclear whether these affects are the result of intrinsic (local thymic milieu) or extrinsic (host environment) thymic factors. Recent studies by Nobori et al. (2006) provide evidence to support the idea that thymic function may be more dependent on extrinsic factors. Using miniature swine model, Nobori and colleagues transplanted thymi from old animals (20–21 months of age) into MHC matched juvenile recipients (4 months old) thymectomized three weeks earlier. Measures of the cortex to medulla ratio (c/m ratio) highlighted a significant difference between aged and juvenile thymi. Following engraftment the c/m ratio improved from initial measurements of 1± 0.3 to 3.1± 0.8 and 4.1± 0.5 at 60 and 100 days posttransplantation, respectively. By day 180 reinvolution was noted with the thymic architecture progressed to resemble that of naïve animal by 240 days. Furthermore, flow cytometric profiles of the thymus from the aged thymic engraftments on day 60 revealed that a majority of the cells were of CD4/

CD8 double positive, CD1 highly positive cells that expressed CD3 poorly. These profiles indicate that these cells are derived from the host-type thymocytes and not graft infiltrating cells. Mackall and coworkers demonstrated that aged mice when given bone marrow transplantation retained the ability to peripherally expand the mature T-cell population at the expense of T-cell receptor diversity. Regarding the regenerative capacity of the thymi approximately 50 % of the peripheral T-cells regenerated post-BMT seen in young mice post-BMT was observed in the aged mice despite a reduction in the overall thymic mass. It further noted that these thymi maintained the ability to negatively select autoreactive T-cell clones (Mackall et al. 1998).

These studies demonstrate that when old thymi are; [1] engrafted into young animals or [2] seeded with bone marrow progenitor cells from young, they can become fully functional. This leads to the question of what age-related changes have occurred to result in the involution of the thymus?

1.6 Age-Related Changes to the Thymus

Age-related thymic involution has a profound effect on naïve T-cell production which ultimately alters the repertoire diversity and composition of the T-cell pool. The underlying cause of thymic atrophy is unknown. Several physiological and pathological factors are known to interfere with the normal function of the thymus which in turn causes the thymus to experience atrophy, these include; infection, disease, ageing, pregnancy, puberty, physical and emotional stress, environmental conditions, alterations in hormonal and cytokine levels as well as deficiency of nutritional factors such as Zinc. A recent publication by Taub and Longo (2005) has reviewed in detailed the contribution made by these factors. Conceivably if the mechanism(s) underlying these changes can be fully elucidated, it may provide strategies whereby the thymus could be rejuvenated with the potential to then increase the immune function in the elderly.

2 Section II: The Impact of Cellular Interactions on Rejuvenation the Age Thymus

2.1 Thymic Rejuvenation

The underlying mechanism(s) that initiate the process of thymic involution are unknown although several physiological and pathological factors, as mentioned above, are known to interfere with the normal function of the thymus and results in atrophy. Unlike age-related thymic atrophy many of the factors are associated with transient or reversible atrophy. This may indicated the extent to which factors within the thymic microenvironment influence the regulation of cellular immunity. Where physiological

resources become limited, for example in the case of Zinc deficiency, the immune system may prioritise first line defense function above more luxurious functions i.e. increasing the T-cell repertoire (Fraker and King 2004; Fraker et al. 2000). Therefore increasing the likelihood of thymic atrophy unless additional signals are received which prevent this process. An alternative point of view regarding the differences between the transient thymic atrophy resulting from these physiological factor and that observed with ageing could be attributed to an accumulation of several of these factors.

Considering the overwhelming impact that thymic involution has on the immune system it is reasonable to hypothesize that if ways can be found to rejuvenate the thymus, thereby increasing its overall function, it may be possible to prevent many of the deleterious effects associated with ageing. Potential factors have been reported to prevent or reverse the thymic atrophy, these include; [1] the action of interleukin 7 (IL-7; Andrew and Aspinall 2001, 2002; Henson et al. 2005; Imami et al. 2000; Phillips et al. 2004; Virts et al. 2006); [2] administration of dietary supplements such as Zinc (Bogden et al. 1990; Fraker et al. 2000; Mocchegiani and Fabris 1995; Prasad 1998), herbal remedies like Ginkgo biloba leaf extract EGb 761 (Tian et al. 2003) and Melatonin (Tian et al. 2001); and (3) the activity of steroidal hormones (Heng et al. 2005; Sutherland et al. 2005). The ability to rejuvenate thymic output is not only beneficial in the context of ageing but also to individuals requiring reconstitution of their T-cell repertoire due to infections (HIV) or following medical intervention (cancer therapies). The major findings of some of the factors currently being studied for thymic regeneration will be discussed (summarized in Fig. 1).

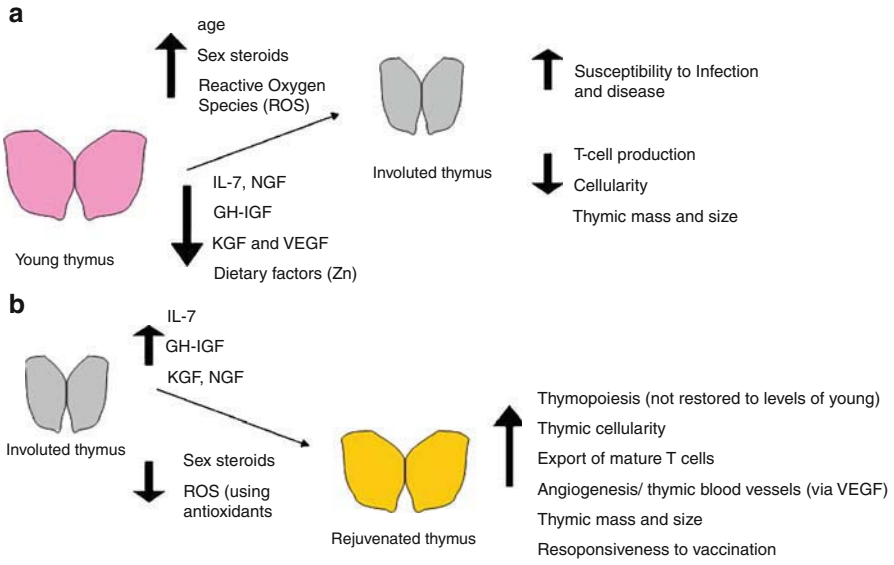

Fig. 1 Factors affecting thymic function and potential methods for thymic rejuvenation (a) Factors including age, sex steroid production and reactive oxygen species implicated thymic involution (b) Administration of a variety of factors associated with a rejuvenation of the thymus

2.2 The Role of IL-7 in the Immune System

IL-7 is a pleiotropic cytokine with a central role in the development and maintenance of T-cells. IL-7 is classified as a type 1 cytokine of the hematopoietin family. The protein can be detected in several tissues including epithelial cells in the thymus (Grassi et al. 2004) and gut (Hansen et al. 2006), stromal cells in the spleen and bone marrow (van et al. 2005), keratinocytes (Moore et al. 1993), fetal (van et al. 2005) and adult liver (Madrigal-Estebas et al. 1997), activated dendritic cells (Gutierrez-Ramos et al. 1992), follicular dendritic cells (Heufler et al. 1993) and also astrocytes (Golden-Mason et al. 2001). In humans and in mice the size of the peripheral T-cell pool is notably constrained between defined limits despite age related changes in thymic output (Michaelson et al. 1996). Such control is achieved partly by IL-7 through homeostatic mechanisms which regulate cell survival and expansion (Roifman et al. 2000). To date, the predicted structure of IL-7 suggests four helices (A–D) are involved in the binding to the heterodimeric IL-7 receptor (Cosenza et al. 2000; Kroemer et al. 1996, 1998; Kroemer and Richards 1996). The IL-7 receptor consists of 2 chains, α (CD127) and a common γ (CD132) which is shared with other cytokines including IL-2 and IL-4 (Kondo et al. 1993, 1994; Noguchi et al. 1993; Russell et al. 1993). Helices A-C binding to the α chain, whereas loop D binds the γ chain. The interaction of helix D with γ chain is vital for the proliferation of T-cells (Page et al. 1993, 1997). Since CD132 is common to many cytokines it is conceivable that the specificity for the IL-7 binding activity resides within the α chain (Kroncke et al. 1996; Sorg et al. 1998) interaction. It is also worthwhile noting that several isoforms of the IL-7 have been describes with slight differences in activity (Kroemer et al. 1998). Reports indicate that the changes to the peptide sequence of the isoforms results in alterations in the structural conformation of the protein due to loss of critical disulphide bonds (Cosenza et al. 2000). The interaction between the IL-7 and IL-7Rα results in the dimerization with the common γ chain, this initiate the phosphorylation of tyrosine residues on IL-7Rα by Jak3 and the recruitment of Jak1 and STAT molecules. The cascade of downstream signaling events results in the survival, proliferation or differentiation of the cell (Fry and Mackall 2002c; Olosz and Malek 2000; Ziegler et al. 1995) See Fig. 2.

IL-7 is critical for the development and function of several components the immune system including; B and T-cell development (Andrew and Aspinall 2001; Bhatia et al. 1995; Goodwin et al. 1989; Namen et al. 1988a, b; Rodewald and Fehling 1998); modulation of T-cell maturation (Gringhuis et al. 1997) partly through the up-regulation of bcl-2 family of molecules (Boise et al. 1995; Fry et al. 2001; Vella et al. 1998) and dendritic cell development (Varas et al. 1998). In-depth review of IL-7 role contribution to the immune system can be found elsewhere (Aspinall 2006; Aspinall et al. 2004; Fry and Mackall 2002c). A role for IL-7 as a potential therapeutic agent to increase the T-cell numbers has been suggested following bone marrow transplantation (Bolotin et al. 1996), in HIV infections (Fry and Mackall 2002b) and rejuvenation of immune system in the elderly (Aspinall et al. 2007).

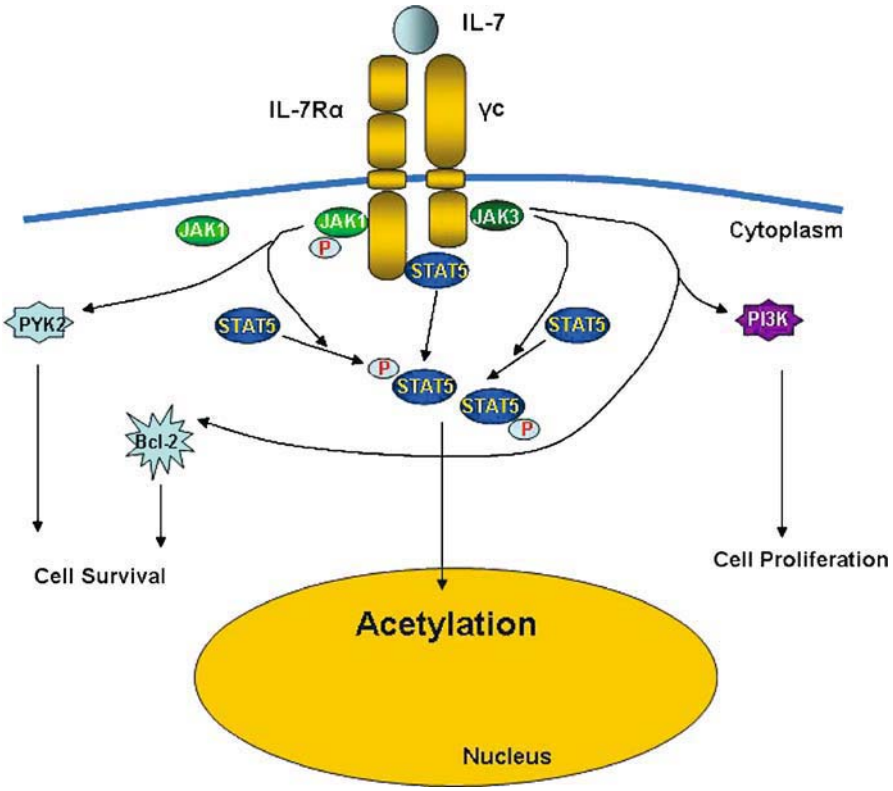

Fig. 2 IL-7 signal transduction pathway. Interaction of the IL-7 and the IL-7 receptor initials a cascade of downstream signaling pathways which results in cell survival or proliferation. IL-7 interactions are thought to be critical for T-cell production and maintenance of the thymic function

2.3 Methods of Thymic Regenerations

2.3.1 Rejuvenation of the Immune Function by IL-7: Studies in Animals

IL-7 is produced in the thymus and bone marrow where normal T-cell precursors develop and studies suggest that the level of IL-7 production may be a critical modulator of T-cell development. Initial studies by Bhatia et al. (1995), on young mice treated with anti-IL-7 showed that severe thymic atrophy occurred with greater than 99 % decrease in thymic cellularity after prolong administration. The similarity between the atrophy seen following treatment with antibodies to IL-7 and that seen in ageing prompted an analysis of IL-7 expression with age in the thymic stromal cells. In the mouse MHC Class II+ epithelial cells have been shown to be the site of IL-7 synthesis within the thymus (Moore et al. 1993). Using quantitative PCR one study has shown that IL-7 levels decreased 15-fold by 22 months of age within the thymus, but that keratin-8, a molecule whose expression is associated primarily

with cortical epithelial cells only showed a sixfold decline by 22 months of age (Ortman et al. 2002). These results echoed an earlier study (Andrew and Aspinall 2002) which showed that the age-associated decline in intrathymic expression of IL-7 was not matched by a similar decline in expression of connexin 43 a molecule associated with gap junction formation in thymic epithelial cells (TEC; Alves et al. 1995). In situations where IL-7 production is absent or reduced thymic atrophy is induced, resulting in normal levels of DN1 population but a reduction in all other developmental stages. This effect is reversed with the addition of IL-7. Conversely, where IL-7 is expressed at excessive levels a similar bottleneck in at the DN1-DN2 developmental stages occurs. In a report by Abdul-Hai, IL-7 administered after syngeneic bone marrow transplantation resulted in a 12-fold increase in thymic cellularity. In addition, RAG-1 expression and V-D-J recombination were increased in IL-7 treated animals (Abdul-Hai et al. 1996). Bolotonin and colleagues showed that the administration of IL-7 after BMT resulted in a more rapid normalization in thymic cellularity and thymic subsets (Bolotin et al. 1996). Furthermore, increased numbers of thymus derived mature T-cells were seen following BMT with IL-7 treatment. Thus, exogenous IL-7 enhances thymopoiesis after radiation induced lymphopenia (Bolotin et al. 1996; Fry and Mackall 2002c).

Work undertaken by Aspinall et al on aged mice has shown that stimulation by IL-7 can reverse age-related atrophy of the thymus, leading to a restoration of thymic output (Andrew and Aspinall 2001; Henson et al. 2005). Phillips et al. (2004) and Virts et al. (2006) have demonstrated that intrathymic injection of IL-7 secreting S17 cells was also capable of preserving high levels of DN2-DN3 thymocytes in old age compared to age-matched controls with an additional observed increase in the expression of bcl-2 levels (Phillips et al. 2004; Virts et al. 2006). These authors also suggest that despite these findings the thymic involution was not diminished with age. One striking features associated with the lack of IL-7 production is the reduced thymopoiesis and export into the periphery. These events may fuel additional complications within the T-cell pool due to the disproportional relationship between the naïve and memory T-cell fractions. Additional studies undertaken in mouse and primates have investigated the rejuvenating effects of IL-7 on immune function. Initial studies by Melchionda et al. (2005) demonstrated that by administering recombinant human IL-7 to mice during immunization against the male antigen HY resulted in an increase in effector cells against subdominant antigens (Melchionda et al. 2005). In a study by Aspinall et al. (2007), it was shown that when old female rhesus macaques aged between 18–24 years (equivalent to greater than 60 years in humans) were treated with recombinant simian IL-7 prior to vaccination with A/PR/8/34, an increase was seen in thymic output as measured by TREC assay (*See* Fig. 3). It was also observed that haemagglutin titre was higher in the animals treated with IL-7 compared to those treated with saline indicating administration of IL-7 had enhanced the immune response (Aspinall et al. 2007).

Snyder et al. (2006) has recently discussed the possibility of using IL-7 therapy in the allogeneic transplantation setting. A major complication associated with hematopoietic stem cell transplantation is obtaining the balance between graft versus tumor effect as opposed to graft versus host diseases (GVHD). While T-cell

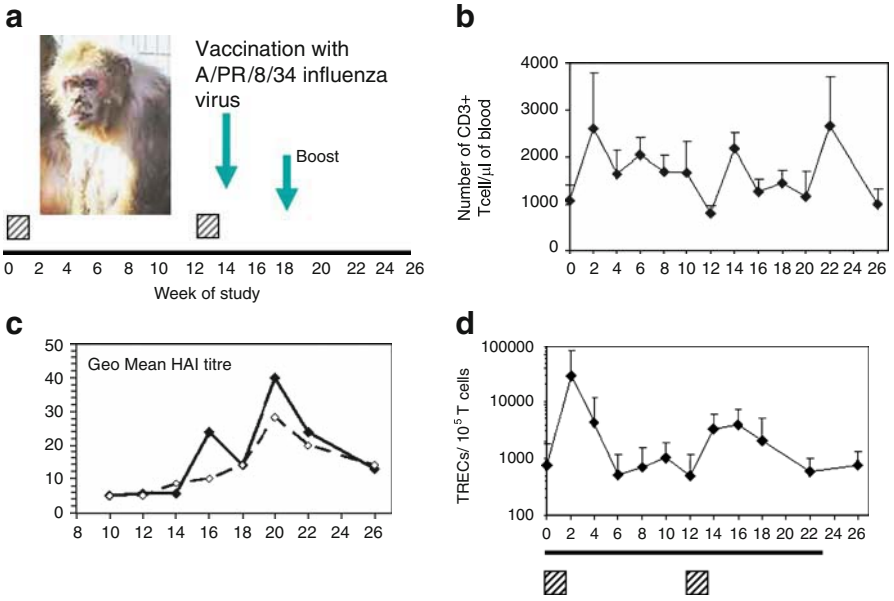

Fig. 3 (a) Schematic of the vaccination strategy in female primate following adjuvant therapy using IL-7 or saline (indicated by shaded box) (b) T-cell numbers as measured by CD3 levels corresponding to week of study (c) Haemagglutination inhibition assay (HAI) demonstrating higher haemaggluttin titre levels in the primates treated with IL-7 (closed circles) compared to saline treated (open circle) (d) Increase in thymic output as measured by T-cell receptor rearrangement excision circle (TREC) assay after IL-7 treatment. Adapted from Aspinall et al. (2007)

depletion can effectively prevent GVHD this increases the risk of graft rejection and along with prolonged lymphopenia and immunosuppressive agents results increases the susceptibility to infection and relapse of malignant disease (Sehn et al. 1999). The ability to reconstitute the T-cell either by generation of new T-cells through the thymus or by the expansion of existing T-cell present from the host or contained in the graft would improve the outcome of the treatment. Therefore pleiotropic nature of IL-7 may provide a promising means of improving transplant outcome by enhancing homeostatic peripheral expansion and perhaps by enhancing reactivity to weak tumor antigens. Initial murine studies have demonstrated a fine balance exists in IL-7 requirement, if too much is given this appears to exacerbate GVHD (Sinha et al. 2002) whereas too little results in no beneficial effect (Alpdogan et al. 2001, 2003).

2.3.2 Studies in Human

The vast majority of IL-7 studies in the literature have been conducted on murine disease models. However, Rosenberg et al. (2006) recently published the finding of a clinical trial in which examined the therapeutic effects of IL-7 administered

to humans with metastatic cancer. Patients were subdivided into four cohorts and each received a total of eight subcutaneous injections at 3-day intervals for 21-day at a given dose of IL-7. The dosage given was 3, 10, 30, or, 60 μg/kg. Increases were noted in the CD4/CD8 lymphocyte ratio at 10, 30 and 60 μg/kg. Interestingly this increase was maintained above baseline values 7 days after the last injection was given at the highest concentration. The immunophenotype indicated an increasing trend towards a higher proportion of naïve relative to memory cell at 60 μg/kg. Analysis of the $CD4^+$ regulatory T-cells as defined by $CD4^+CD25^+FoxP3$ demonstrated a decrease in expression of these cells both before and after IL-7 administration indicating that the observed IL-7 mediated expansion in the $CD4^+$ and $CD8^+$ T-cells was restricted to selective population of T-cells. A noteworthy observation was that a proportion of these cells did not express the IL-7 receptor (CD127) which may account for the nonresponsiveness to IL-7 therapy (Rosenberg et al. 2006). The IL-7 formulation used in this study was nonglycosylated which therefore had the potential to generate immunological side effect at higher doses, further highlighting the need for safer alternative therapeutic agents. In addition this study clearly identifies the potential therapeutic benefits to be achieved using compounds that mimic the function of IL-7.

Taken together these results may have implication on the methods used for regenerating the thymus or suggest that additional factors may be required to truly reverse age-related these changes. It may also reflect a deeper level of complexity in the development of thymocytes than simply replacement of a single factor.

2.4 Growth Factors, Hormones and Sex Steroids

2.4.1 Growth Factors

Growth factors are an enormous group of diffusible molecules involved in regulating cell survival, differentiation, and proliferation. Each growth factor binds specific cell surface receptors, activates them, and induces intracellular signal cascades ultimately leading to characteristic cellular responses (Garcia-Suarez et al. 2003; James and Bradshaw 1984; Sporn and Roberts 1992; Yarden and Ullrich 1988). Among them, some have proven necessary to some extent to sustain thymic function, these include; Keratinocyte growth factor (KGF), nerve growth factor (NGF) and insulin growth factor I (IGF-I).

2.4.2 KGF

KGF is a member of the acidic fibroblast growth factor receptor 2 family and is produced by fibroblasts and many mesenchymal cells (Rubin et al. 1989). Its mitogenic effects have been shown to stimulate proliferation and differentiation in a variety of tissues and functions as a growth factor for epithelial protection and repair

in various forms of injury and tissue damage (Adamson and Bakowska 1999; Baskin et al. 1997; Ichimura et al. 1996; Marchese et al. 1995). Several studies have demonstrated KGF is necessary for fetal and postnatal thymic epithelial development (Alpdogan et al. 2006; Jenkinson et al. 2003). Alpdogan et al. 2006 demonstrated that the administration of KGF enhanced T-cell development and reconstitution following irradiation induced thymic damage and in mice as old as 18 months. Interestingly, thymic involution was not accelerated in KGF deficiency mice, despite its effects on thymopoiesis (Alpdogan et al. 2006). Rossi and colleagues investigated the cellular mechanism whereby KGF stimulates thymic T-lymphopoiesis in adult mice, their findings indicated that the KGF- specific receptor, FgfR2IIIb (fibroblast growth factor receptor 2IIIb) is expressed on mature cortical and medullary as well as immature TEC. Exogenous exposure to KGF results in proliferation and expression of several growth factors including Wnt5b, Wnt10b, along with BMP2 and 4. Consequently, an increase in thymopoiesis commencing with the most immature T-cell precursors and leads to an increase in thymic cellularity and enhanced export of mature T-cells to the periphery (Rossi et al. 2007). Rossi et al. suggest that treatment with KGF affects the microenvironment allowing for larger developmental niches in which to accommodate increased amounts of early stage thymocytes required for thymopoiesis. A transient decline in the number of T-cell precursor homing and entering into the thymus is seen by a decreased CCL25 expression within the TEC. This may be associated with a qualitative maturation process, as the thymocyte undergo a transition from TN1 to mature thymocytes. By preventing T-cell precursors entry this provides the time necessary for this process (approximately 15-day; Rossi et al. 2007).

Interestingly, a controversial connection between IL-7 and KGF has been proposed by Min et al. 2002, who have suggested that increased thymopoiesis by KGF expression is dependent on IL-7 (Min et al. 2002). Observation by Alpdogan et al. 2006, makes the case that these increases are due to phosphorylation of STAT-3 and not IL-7 related STAT-5 possibly indicating that thymocyte development via KGF expression is through an alternative pathway (Alpdogan et al. 2006).

2.4.3 NGF

NGF is produced by medullar TEC and binds to the high affinity neutrophin receptor, tyrosine kinase receptor A (TrkA; Kaplan et al. 1991). NGF is involved in mechanisms related to the modulation and regulation of immune cell proliferation, development, differentiation and activation (Aloe et al. 1997; Lee et al. 2007; Vega et al. 2003). Interestingly, it has been demonstrated that in rats during thymic regeneration following acute thymic involution induced by cyclophosphamide, TrkA is upregulated in the thymic subcapsular, paraseptal, perivascular, cortical epithelial cells and medullary epithelial cells including the Hassall's corpuscles (Lee et al. 2007; Yoon et al. 2003). Additionally, NGF mRNA and protein were expressed in unstimulated thymocytes, with the expression increasing during thymic regeneration. The involvement of NGF-TrkA interactions in thymic regeneration following acute involution

is clearly evident, but is the same true for age-related involution. Previous studies have found that decreasing levels of NGF and TrkA with increasing age providing a possible link to the changes in appearances observed in the thymus (Garcia-Suarez et al. 2000). Turrini et al. (2001) observed that aged mice treated with NGF caused a significant increase in the number of thymocytes and prevention of thymic cell death suggesting a role of NGF in maintaining thymocyte viability (Turrini et al. 2001). Finally, a recent study by Park et al. 2007 has demonstrated a link between NGF/TrkA levels and reparative angiogenesis through vascular epithelial growth factor. VEGF was shown to colocalize in the TrkA positive TEC. When subcapsular nurse epithelial cells were directly stimulated in vitro with NGF, VEGF mRNA and protein levels were shown to increase. In vivo injection of NGF also caused an increase in VEGF protein and elevated thymic blood vessel. These results indicate that NGF promotes the production of thymic VEGF in vitro and in vivo (Park et al. 2007).

2.4.4 IGF-I

IGF-I plays an important role in thymocyte proliferation and survival. The IGF-I receptor is located on thymocytes, whereas IGF-I is secreted from macrophages with expression levels of IGF-I steadily declining with age in rodents and humans (D'Costa et al. 1993; Lamberts et al. 1997). Administration of IGF-I to cyclosporine treated or diabetic rats and mice results in an increase in cellularity and thymic size (Beschorner et al. 1991; Dorup and Flyvbjerg 1993; Montecino-Rodriguez et al. 1998; Tian et al. 1998). Given the mitogen and anti-apoptotic effects, shortage of IGF-I could contribute to the thymic involution and therefore may potentially be used to rejuvenate the thymus. A limiting factor to the effectiveness of IGF-I to rejuvenate the thymus is that thymocyte production is never restored to levels of the young animal. Furthermore, IGF-I treatment does not significantly altered single positive distribution in mice or primate studies (LeRoith et al. 1996).

2.5 Growth Hormones and Sex Steroids

It has been a long established view that alterations in the ratio of growth hormones to sex steroids are important factors in thymic atrophy. The presence of increasing levels of sex steroids, marking the onset of puberty, has been linked with thymic atrophy (Hirokawa et al. 1994; Utsuyama et al. 1995). When chemical or surgical castration is performed on aged animals, regeneration of the thymus is observed. These effects can be reversed by the administration of synthetic sex steroids (Fitzpatrick and Greenstein 1987; Fitzpatrick et al. 1985; Greenstein et al. 1986, 1987; Kendall et al. 1990). Sex steroids act on early thymocyte differentiation, specifically blocking the triple negative stages 1 to 2 (TN1 to TN2 stage; Aspinall 1997; Heng et al. 2005; Thoman 1995). Progression through the TN development stages is IL-7 dependent and therefore suggests that the castration affects may be mediated by IL-7 (Heng et

al. 2005). A recent report by Min and colleagues (Min et al. 2006), investigated the validity of the hypothesis that low levels of growth hormones (GH) and high sex steroid production accelerate thymic involution. The authors used mice with mutations in the genes encoding for the growth-hormone-releasing factor receptor or gonadotropin-releasing hormone, which leads to a reduction of GH and diminished sex steroid production (Min et al. 2006). The results indicated that changes in the production of GH or sex steroids were not required to initiate or sustain thymic involution. In addition by blocking the sex steroid production did not delay thymic involution. These results are contrary to the finding of other groups which have shown increase in thymic cellularity following castration. It is suggested by Min et al. that these cellular effects are transient and that the thymus still undergoes involution.

An interesting development in recent years is the discovery of a class of synthetic nonpeptidyl compounds known as Growth Hormone secretagogues (GHS). These compounds have the ability to synergize with natural GH-releasing factor and have been shown to induce calcium flux within rat pituitary cells which causes the release of GH. The full description of the discovery process and development of GHS can be found elsewhere (Smith 2005; Smith et al. 2005). Koo et al. demonstrated that when 5-6 week old B6 mice were given 5mg/kg of GHS orally for 3 weeks they experienced a 30 % increase in lymphoid cells in the peripheral blood compared to control mice. Further experiment on 20–24-month-old BALB/c or B6 mice given oral doses of GHS old mice did not show an increase in white blood cell numbers or have any affects on T- or B cell proliferation. However, a significant increase was observed in thymic cellularity in the treated animals that was consistent across all thymic subsets. Similar observation was seen when 16-month-old mice were treated with 1mg/kg i.p. from Monday to Friday for 3 weeks. In order to determine the effect of GHS in a disease model the authors examined the effects of GHS in resistance to a transplantable tumor, EL4 (H-2^b, derived from B6 mice). EL4 is an aggressive tumor in syngeneic B6 mice, causing mortality in 3–5 weeks. All animals treated with GHS for 3 week prior to inoculation showed a significant decrease in metastases associated with EL4 tumor development compared the untreated controls demonstrating an enhanced host mediate response. This observation was seen in all ages of mice tested (16–24 months). The overall findings indicate an enhancement of immune function by the regeneration of thymic cellularity and also the increased resistance to tumor metastasis in mice (Koo et al. 2001).

The potential for using GHS in humans has clear advantages as demonstrated from data regarding the treatment of individual with growth hormone deficiency. When GH is used, particularly in the elderly it is reported to result in an increase in the IGF-I levels, lean body mass and spinal bone density and a decrease in fat mass in men older than 60 years (Marcus et al. 1990; Papadakis et al. 1996; Rudman et al. 1990). With these beneficial effects associated with GH treatment in the elderly and other groups including individuals with HIV and cancer sufferers (Mackall and Gress 1997), GHS offers several advantages. For example, physiological GH release in the elderly is pulsatile which can lead to supraphysiological levels being experienced from high doses of GH; dependent on the pharmacodynamic and pharmacokinetic properties of the GHS being used this can be overcome. Studies performed with an

orally active nonpeptidergic GHS (MK0677) resulted in a significant increase in IGF-I in individuals over 60 years old compared to placebo groups after 4 weeks treatment (Chapman et al. 1996; Nass et al. 2007). Overall, GHS were well tolerated and their use was safe (Bach et al. 2004).

Similarities between the effects of GH and IGF-I is likely to result from the close interaction between them. GH induces IGF-I and is thought to mediate a number of GH actions, therefore despite the obvious involvements of IGF-I and GH, treatment with either alone is unlikely to be sufficient to rejuvenate the involuted thymus (Olivia García-Suárez 2003; Taub and Longo 2005).

2.6 Dietary Supplements

2.6.1 Zinc

Several dietary supplements have been suggested as potential boosters for the immune system. Zinc deficiency has been identified in a number of disorders the most notable including sickle cell anaemia and acrodermatitis enteropathica. Individuals suffering from Acrodermatitis enteropathica, an autosomal recessive disease caused by a defect in zinc metabolism, experience thymic atrophy and impaired cell-mediated immunity resulting in increased susceptibility to infection and disease (Oleske et al. 1979). These symptoms are effectively corrected by supplementation with zinc.

There are several interesting factors associated with Zinc which warrant further investigation to elucidate its contribution to cellular immunity. Firstly, a hallmark of zinc deficiency in animal models is the development of age-independent thymic atrophy (Prasad 1985). Secondly, individuals with zinc deficiency are known to suffer from increase susceptibility to infection and disease indicative of poor immune function. Third with increasing age there is a decreased ability to absorb Zinc in the gut therefore increasing the likely of individuals become deficient of Zinc (Fraker and King 2004). Fourth, studies in aged mice have shown that drinking water supplementation with zinc sulphate can increase thymic mass and possibly thymopoiesis (Fraker and King 2004). Fifth, Zinc deficiency has been noted as a secondary condition in disorders such as diabetes, AIDS, Down's Syndrome and select cancers (Keen and Gershwin 1990). Sixth, Zinc supplementation has been shown to increase thymulin secretions in aged mice (Mocchegiani and Fabris 1995) and human (Prasad et al. 1988) suggesting a beneficial role for thymic function. Collectively these factors provide compelling reasons for investigating the potential impact to be made by Zinc on the immune system of free living old people.

2.6.2 Ginkgo Biloba Leaf Extract EGb 761

Ginko biloba leaves have been used as part of traditional Chinese medicine for several thousand years. EGb761 is the complex chemical mixture extracted from the Ginko biloba leaf and has been shown to have protective and rescue effects

on a variety of medical conditions including neurodegenerative disorders (Ramassamy et al. 1999), cardiovascular disease (Pietri et al. 1997) and ageing (Winter 1998). The functional properties of EGb761 have been attributed to its antioxidant and free radical scavenging activities. Tian and colleagues demonstrated that by administration of EGb761 both in vitro and in vivo was capable of protecting thymocytes against the reactive oxygen species. Oral dosage of EGb761 was given for 60 days at 1,600 μg/day/mouse to 22 month old C57BL animals. After this time, the mice were sacrificed and the size of their thymus and spleen were assessed. It was found their organs had significantly increased in mass compared to untreated age-matched controls. These mice were also observed to have significant responsiveness to mitogens (Tian et al. 2003). Similar results were obtained when investigating the effects of melatonin which also known to act on reactive oxygen species (Tian et al. 2001). This suggests that compounds which antioxidants may also be important for the rejuvenation of the thymus.

These findings highlight the complexity facing those investigating the restoration/ rejuvenation of thymic function. It is unlikely that any single factor will be found capable of restoring thymic function but more conceivable that a combination of the mechanisms describe will all be required to make a functional contribution. So far, we have examined physiological and dietary factors as the means of understanding the triggers of thymic involution and how by using our understanding of these systems we can devise potential strategies for immunorejuventation. Aside from these factors, alternative approaches aimed at utilizing the current knowledge of the cellular interactions are emerging and are providing interesting data highlighting the potential of generating and targeting of specific T-cells to bolster the immune response. Several of these will be discussed.

3 Section III: Methods for Generating Functional T-Cells Ex Vivo

3.1 Thymus Independent T-Cell Development

The majority of T-cell development is known to occur within the thymus, and although recent description of extrathymic T-cell have been reported in oncostatin M (OM)- transgenic mouse, it is generally considered that these T-cells do not function in the same way as their thymus derived counterparts (*See* review by Blais et al. 2006). The question therefore remains, 'Can functional T-cell be developed anywhere other than the thymus?'

Over the past decade studies focused on addressing this question have provided encouraging results in favor of in vitro T-cell development. In vitro systems including fetal thymic organ cultures (FTOC) and reaggregate thymic organ cultures (RTOC) have been devised and studied, and are providing valuable contribution to our understanding of T-cell differentiation, and positive and negative selection processes (Hare et al. 1999). Both systems are able to support the development of all

T-cell subsets. However, drawbacks included time-consuming nature of the procedure and expense for a relatively low cellular yield. The alternative in vitro approach using thymic stromal cell monolayer (TSMC) also had a major problem in that these system originally displayed an inability to progress beyond the DN1 ($CD4^-CD8^-CD44^+CD25^-$) stage of development. So, on the one hand, FTOC and RTOC can sustain T-cell development whereas TSMC could support limited progression. These differences between the two approaches were initially attributed to a requirement for a 3-dimensional architecture as a means of providing the microenvironment needed to resemble that within the thymus. Two crucial studies provided vital insight into T-cell development. First was the observation that Notch 1 deficient hematopoietic progenitor cells fails to give rise to T-cells and instead developed into B cells in thymus of mice and secondly that BM cells transduced with a portion of intracellular Notch domain developed into double positive T-cells in the bone marrow clearly implicating Notch signaling with a central role in T-cell development (Pui et al. 1999; Radtke et al. 1999). With the critical discovery of Notch signal involvement in T-cell development, Zuniga-Pflucker and coworker generated the OP9-DL1 coculture system (*See* de Pooter and Zuniga-Pflucker for a review of the OP9-DL1 approach (de Pooter and Zuniga-Pflucker 2007)). The OP9 cell line, is derived from a macrophage colony stimulating factor (m-CSF) deficient mouse, m-CSF is required for myeloid lineage proliferation, therefore making OP9 cells particularly suited for studying lymphocyte development. The OP9 cell line transduced with Notch ligand Delta like-1 (DLL1) or Delta like ligand 4 (DLL4) resulted in the OP9-DL1 and OP9-DL4 cell lines. The OP9-DL1 cell line could support T-cell development in a monolayer culture due to the expression of the Notch ligand delta like ligands (DLL1 or DLL4; Schmitt and Zuniga-Pflucker 2006) when seeded with numerous progenitor cell populations (De Smedt et al. 2004; Hoflinger et al. 2004; La Motte-Mohs et al. 2005; Porritt et al. 2004; Schmitt et al. 2004a, b). These data suggest that the delta-like family of Notch ligands collectively acts to induce T-cell commitment and differentiation in the thymus (Schmitt and Zuniga-Pflucker 2006). Mohtashami and Zuniga-Pflucker further demonstrated that the essential difference between FTOC, RTOC and TSMC was the ability to maintain expression of DLL1 and DLL4 (Mohtashami and Zuniga-Pflucker 2006). Ectopic expression of DLL1 and DLL4 in TSMC was capable of restoring T-cell development in the absence of a 3D microenvironment. The OP9-DL1 system has altered the original view that monolayer cultures were incapable of generating T-cell and provided a simple method for studying the molecular mechanisms that control early T-cell development. A limitation to the OP9-DL1 systems is the inability to mediate positive and negative selection and therefore will require the development of additional systems.

An alternative approach to answer the question of, in vitro T-cell development has been the use of 3-dimensional matrix structures to recreate the cellular microenvironment of the thymus. Originally devised by Poznansky et al. 2000, they demonstrated using murine thymic stroma seeding with human stem cells that within 14 days fully functional $CD3^+$ T-cells could be reproducibly generated (Poznansky et al. 2000; Robertson and Poznansky 2003). The study by Clarke et al. 2005 extended this work further. By focusing on the similarities that exists between the constituent

components found within the thymus and that of the skin, Clarke determined to test the hypothesis that cellular elements of the skin, reconfigured in a different 3-dimensional (3D) arrangement can support the differentiation of T-cells from hematopoietic precursor cells (HPC; Clark et al. 2005). Using Cellform, which is a 3D tantalum-coated carbon matrix originally designed as an artificial bone matrix (*See* Fig. 4), Clarke proceeded to recreate the thymic microenvironment by combining individually cultured fibroblast and keratinocytes cells on the 3D matrix. After 5-6 days, the established keratinocyte/ fibroblast coculture was seeded with isolated $AC133^{+}$ hematopoietic precursors derived from human bone marrow in the presence of a cocktail of cytokines including IL-7, IL-15 and Flt-3 ligand. T-cells were generated from the 3D thymic construct when keratinocyte only or keratinocyte/fibroblasts were seeded with AC133 cells. The most robust production was observed with the keratinocyte/fibroblast combination. In addition to T-cells, $CD14^{lo}HLA\text{-}DR^{hi}$ DCs,

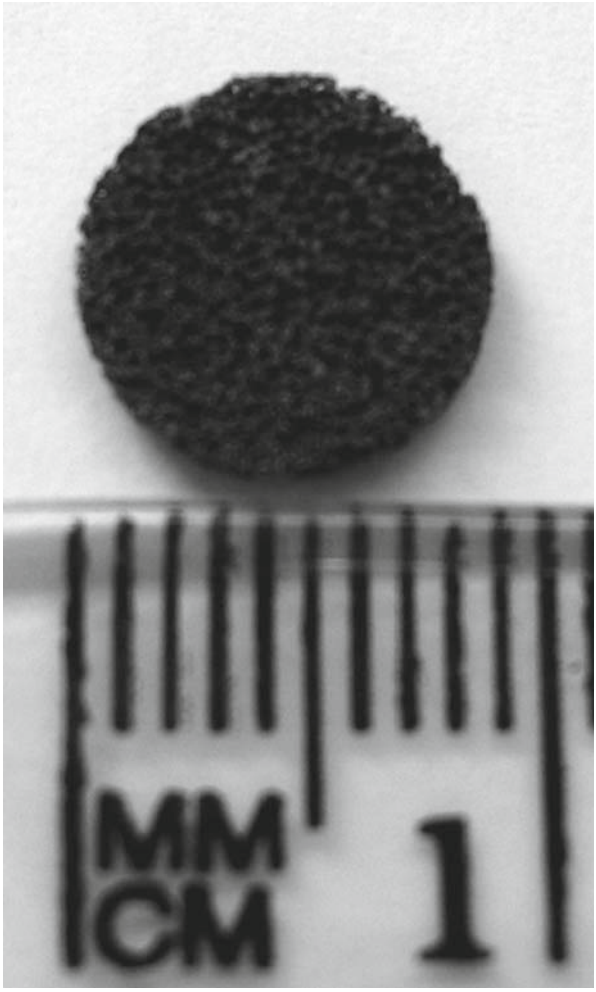

Fig. 4 Cellfoam 3 -dimensional tantalum-coated carbon matrix used for the generation of T-cells ex vivo as described by Clark et al. (2005)

$CD14^+$ myeloid cells and variable numbers of $CD56^+$ cells were also produced. The T-cells generated were shown to consist of 95 % $CD3^+$ αβ-TCR, from which T-cell receptor rearrangement excision circles (TREC) could be measured, therefore confirming that the T-cell had arisen from newly generated T-cells and not as a result of expansion of contaminating T-cell from the original seeding process. Positive and negative selection are critical for T-cell maturation and function therefore the appearance of double positive $CD4^+/CD8^+$ T-cell precursors before the mature single positive CD4 and CD8 T-cells, supported the notion that the T-cell construct was capable of positive selection. Mixed leukocytes reactions using autologous and allogeneic derive DC further demonstrated that the construct derived T-cells responded in the allogeneic but not autologous DC. These results suggested the existence of the negative selection of autoreactive T-cells. Importantly the analysis of the T-cell repertoire provided additional evidence of newly generated T-cells as the spectratype profile was different from the donors. Finally to address the question of whether the construct derive T-cells were functional and capable of proliferation, they were stimulated with mitogens and alloantigens and proliferated in response to phytohemagglutinin. These T-cells were found to express robust levels of CD69 (an early activation marker) in response to stimulation with concanavalin A (Clark et al. 2005). A Notch delta-like ligands, which is vital for T-cell production was expressed on the keratinocyte of the skin culture and may partially explain the ability of the culture to support T-cell development. In addition, the skin culture also expressed *Foxn1* and *Hoxa3* which are required for the differentiation of epidermal keratinocytes and promote migration of cell required for the development of the thymus.

4 Section IV: Adoptive Transfer of T-Cells as Applied in the Clinic

As described previously, the decline in T-cell numbers is associated with ageing and affects the immune competency of the individuals due to infections and disease. The ultimate aim is to provide a means of bolstering the T-cell mediated immune response to restore a fully functional immune system. Currently we have discussed a range of factors that effect the thymus and also two different approaches to address the challenge of ex vivo T-cell development. However, as a consequence of our increased levels of knowledge, more exciting new questions arise. In the final section we will examine the potential benefits offered by adoptive transfer of T-cells as applied to combating Cytomegalovirus (CMV) infections. Adoptive transfer is based on the principle of isolation and infusion of antigen specific or nonspecific lymphocytes with the aim of replacing, repairing or enhancing immune function primarily in the stem cell transplantation setting (Porter and June 2005). For reviews examining the adoptive T-cell therapy for cancer the readers is referred to recent papers by Carl June (June 2007a, b). Parallels exist between the immunological state of the SCT patient and elderly individuals which render both at increased susceptibility to infection; adoptive transfer in the SCT arena has proven to be beneficial

in reducing treatment related morbidity and mortality. Therefore can an adoptive transfer approach be employed to aid the capacity of the ageing immune system to combat CMV?

4.1 CMV in the Elderly

The CMV is a member of the Herpes viruses and belongs in the subfamily of beta herpes viruses and is known to display, among other characteristics, the ability to undergo periods of latency before reactivation resulting in persistent recurrent infections. In healthy adults CMV infection is mostly asymptomatic although it can result in malaise, fever, sweats and abnormal liver function (Wreghitt et al. 2003). It is generally recognized that CMV infection results in life long infection which is kept under control by immunosurveillance from rapidly established memory T-cells. An increasing body of literature has emerged reporting on the impact of infection by CMV on the ageing immune system (Looney et al. 1999). Data from the Swedish longitudinal studies, OCTO and NONA, examined a variety of immunological parameters in 80–90-year-old individuals to determine whether predictive changes associated with between 2 and 4-year survival could be identified. An increase in seropositivity for CMV was included as a "high risk" predictive factor. Collectively the identified factors are defined as the "Immune Risk Phenotype (IRP)" (Olsson et al. 2000; Wikby et al. 2002, 2005). Akbar and Fletcher postulate that the impact of CMV on the elderly may arise from a competition for space within the T-cell pool with an increase of CMV specific T-cell at the expensive of other memory T-cells. Therefore the IRP associated increases in CMV seropositivity, may correlate to the loss in repertoire diversity and hence reduced ability to combat other infections (Akbar and Fletcher 2005). This argument is supported by evidence that CMV seropositivity correlates to a reduction in EBV-specific (Khan et al. 2004), Influenza-specific (Trzonkowski et al. 2003), T-cells and an increase in CMV CD8 specific T-cell clonality (Khan et al. 2002). Furthermore, it has been shown to accelerate the decline in the naïve T-cell composition and increase the number of cells of $CD28^-$ cells in CMV-infected individuals of all ages (Almanzar et al. 2005). In addition cognitive decline has also been over in elderly individuals with high levels of CMV antibodies (Aiello et al. 2006). In light of these findings, strategies that can reduce the CMV burden on the ageing immune system may prove useful in reducing the susceptibility to other infectious agents.

Besides the effects noted above affecting elderly populations, CMV status is an influential factor for treatment related complications in immunocompromized individuals such as stem cell transplant recipients. Within this setting, CMV positive recipients who received donation from CMV negative donor required increased levels of ganciclovir treatment as the CMV-specific CD8 responses were delayed compared to when both donor and recipient were CMV positive (Aubert et al. 2001). In 2005, Cobbold et al. used adoptive transfer of donor derived CMV-specific $CD8^+$ T-cells isolated from the blood as a means of combating reactivation of CMV in SCT

patients. Previous studies had shown that the isolation of CMV-specific $CD8^+$ T-cell clones from the donor that were cultured ex vivo and then transferred to the recipient were effective in the prevention of reactivation by CMV in those individuals unresponsive to antiviral therapy (Einsele et al. 2002; Peggs et al. 2001; Riddell et al. 1992; Walter et al. 1995). In the Cobbold study CMV-specific $CD8^+$ T-cells were isolated using magnetic beads conjugated to HLA-peptide tetramers designed to one of the viral epitope from the pp65 protein (a virion tegument protein and the main component of the envelop particle). This enabled the isolation of the CMV-specific $CD8^+$ T-cells in a "closed" system without the need for ex vivo manipulation (Cobbold et al. 2005). Of the nine patients in the study, no adverse effects were reported and it was found that doses between 1.2×10^4 and $2 \acute{} 10^6$ were sufficient to control and thereby prevent viral reactivation (Cobbold et al. 2005). These results are encouraging as they demonstrate an approach that could be utilized in elderly individuals to combat CMV reactivation. It is conceivable that intervention by infusion of CMV-specific $CD8^+$ T-cells at the point where IRP linked increases in CMV seropositivity is evident may prolong the immunological competence of the elderly.

Akin to the T-cell adoptive transfer approach is the option of using "suicide" genes in which the infused T-cells are modified, to enable effective functional control thus improving the safety and efficacy of the therapy. Suicide genes code for enzymes that render cells sensitive to otherwise nontoxic prodrugs (Cohen et al. 1999; Moolten 1994). An example of this approach is the use of Herpes Simplex virus–thymidine kinase gene (HSV–TK) in T-cell clones, although other including caspase-9 have also been reported (Straathof et al. 2005). HSV-TK converts nucleoside analags such as ganciclovir into monophosphate form, the resultant conversion into triphosphate metabolite causes an inhibition of the DNA elongation leading to cell death (St Clair et al. 1987). Several studies have employed this method as a means of preserving anti-infectious capacity of transferred T-cells while preventing the development of GVHD. The results suggest that this approach when used in the correct way can provide a means of preventing a broader pathogen spectrum compared to antigen-specific T-cell transfers (Andre-Schmutz et al. 2004; Bonini et al. 1997; Bordignon et al. 1995; Marktel et al. 2003; Tiberghien et al. 2001).

5 Concluding Remarks

In conclusion the age-related decline in immunological function provides many challenges for societies in which life expectancy is steadily increasing. In trying to understand ways of rejuvenating the immune systems in older individuals it is becoming clearer that the physiological changes in the levels of cytokines, growth factors and pathological changes such as CMV in status, dietary intake and environmental exposure all contribute to how effectively the immune system functions. Several factors including IL-7, KGF and sex steroid ablation are currently taking centre stage as potential immune rejuvenators. The current strategy of bolstering the immune response by altering the microenvironment in which thymocytes can

develop using the individual factors stated has been beneficial but may provide only limited success; as the interplay and regulation that exist between them becomes more apparent. Strategies that combine two or more of these factors will impart greater insight into the best way of promoting thymic rejuvenation.

A critical feature of immunosurveillance, in the elderly, is the composition and diversity that exist within the T-cell pool. CMV infection is known to affect the dynamics and reduce the number of pathogen-specific memory T-cells thereby resulting in an increased susceptibility to infection by opportunistic pathogens. Every effort is being made to generate fully functional T-cells ex vivo. It will be interesting to see whether ex vivo generated T-cells primed with antigen-specific peptides, for example CMV, will provide the regulatory control needed to enable the maintenance of diversity in the T-cell pool. If ex vivo generated T-cells both primed and naive can be infused into immunocompromised individuals it may then be possible to control the infectious agent (i.e., CMV, EBV) sufficiently to enable an expansion in the number of naïve T-cell assisted by the cocktail of thymic rejuvenating factors. Many challenges questions must still be answered before successful rejuvenation of the immune system can be achieved.

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Assessment of Age-related Decline of Immunological Function and Possible Methods for Immunological Restoration in Elderly

Katsuiku Hirokawa, Masanori Utsuyama, Yuko Kikuchi and Masanobu Kitagawa

Contents

Abstract: The immune system plays an important role in protection against infection and in the maintenance of the internal environment of the body. However, such important immune functions are known to decline with age in many mammals, including humans. It is a matter of clinical importance that the incidence of various age-associated diseases such as infections, cancer and vascular disorders increases with a decrease in immunological vigor. The extent of immunologic decline is variable and exhibits wide inter-individual variations. Thus, it is important to assess the extent of immunologic decline in both patients suffering from various diseases and in healthy people in order to maintain healthy conditions. To this end, we have developed a scoring system that analyzes immune parameters according to a database of known age-associated immune changes obtained from a healthy population. Using this scoring system, we can combine several different immunological parameters and express the immune status of individuals as a simple numeral.

K. Hirokawa (✉)
Institute for Health and Life Sciences
Ascent Myogadani 4F, 4-4-22, Kohinata
Bunkyo-ku, Tokyo 112-0006, Japan
E-mail: hirokawa@h-ls.jp

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After determining immunological vigor for individuals, it is necessary to replenish immune defects and restore them to normalcy for individuals with depressed immunological scores. This chapter provides methods of immunological restoration in animal models and introduces some similar attempts in humans. The effect of any immunological restoration varies with the individual and must therefore verified. Currently, the proposed immune scoring system proposed is useful to determine whether the methods employed are effective for the restoration of immune functions.

1 Introduction

A decade ago, centenarians used to appear mainly in fairy stories. Nowadays centenarians are not uncommon and there are more than 30,000 centenarians in Japan. In 1900 when Soseki Natsume, a Japanese writer, was in London, the mean life span of the male population in England was 44 years old and that in Japan was 45 years old. In many countries, the mean life span has increased steadily during the first half of the 20th century, however, this has not been the case in Japan. The increase in the mean lifespan was outstanding in the second half of the 20th century (after the Second World War) in Japan. This pronounced improvement is attributed to rapid progress of medicine including antibiotics and sufficient food supply including the supply of proteins.

WHO reports that a "healthy" life span is generally 6–10 years shorter than the mean lifespan. This means that many elderly people suffer from some diseases for several years before death.

It is well known that the incidence of cancer, cardiovascular disease, neurovascular disease and infection increases with age. Autopsy examinations have revealed that the largest cause of death in the elderly is infections such as bronchopneumonia and urinary tract infection (Table 1a and 1b). The occurrence of severe acute respiratory syndrome (SARS) in south Asia and China in the winter of 2003 clearly indicated that the fatality rate was high, approximately 50%, in people over 65 years of age (Table 1c) (Hirokawa et al. 2006). With regard to infection, Pawelec et al. (2004) suggested that chronic antigenic stimulation could lead to an increased prev-

Table 1a Causes of death in a hospitalized geriatric population: an autopsy study of 3000 patients

Bronchopneumonia	42.9%
Malignant neoplasms	28.1%
Pulmonary thrombo-embolism	21.2%
Acute myocardial infarction	19.6%
Urinary tract infection	12.3%
Acute cerebrovascular disease	6.5%
Internal hemorrhage	5.5%
Congestive cardiac failure	3.3%

The data, based on 3000 consecutive autopsies (1758 females/1242 males: mean age 80.3 years) performed from 1972 to 1992 in Geneva Geriatric Institutions (Mac Gee W).

Table 1b Major causes of death in autopsy cases of elderly persons at Tokyo Metropolitan Geriatric Hospital

	People over 60 years	People over 70 years
Infections	39.2%	27.6%
Vascular diseases in brain and heart	29.7%	43.1%
Malignancies	18.7%	22.4%
Others	12.4%	6.9%

The data are based on 923 autopsy cases (570 females/353 males) of people over 60 years of age.

alence of senescent dysfunctional T-cells, and therefore contribute to more general alterations in the immune system.

Furthermore, elderly people face an indefinite number of problems such as pain in the shoulder, arm or leg, dizziness, staggering and headaches. In addition, chronic inflammatory symptoms such as bronchitis, laryngitis or adenoiditis are very common in the elderly.

An age-related increase in various diseases is causally related with the age-related decline of immune functions (Hirokawa et al. 2006). Indefinite problems are attributable to variable causes including various diseases and psychological stress, but one of the major causes is inadequate adaptation to variable stresses from the external environment. Nervous, endocrine and immune systems work together to maintain the internal environment, when the body is exposed to the stresses from physical, psychological and biological surroundings. In the elderly, however, the function of all these three systems declines with age, resulting in improper adaptation to stress.

Therefore, restoration of immunological function would be quite helpful for the elderly not only to prevent infection, but also maintain their internal environment at the time of exposure to stress. In other words, immunological restoration is expected to be effective for the improvement of the QOL in the elderly.

Immunological restoration requires 2 steps. The first is to assess immunological parameters or functions to determine the extent of the age-related decline of immune functions of each individual. To this end, we require a measuring method to express the level of immunological vigor as a simple numeral that anybody can understand. The second is to select adequate methods to restore immunological functions and to check the effectiveness of the selected immunological restoration by the measure-

Table 1c Fatality rate of SARS in Hong Kong

Age	Fatality rate (%)
24 years and below	0%
25–44 years	6%
45–64 years	15%
65 years and above	52%
Total	14–15%

WHO report. Consensus document on the epidemiology of severe acute respiratory syndrome (SARS). 17 October 2003, http://www.who.int/csr/sars/guidelines/en/

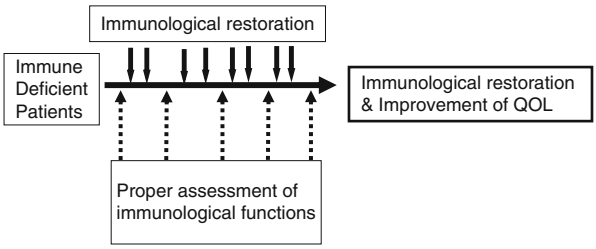

Fig. 1 Proper assessment of immunological functions is essential to verify the effectiveness of immunological restoration

ment method mentioned above, because the effectiveness of immunological restoration differs based on the individual and the method of restoration (Fig. 1). Thus, this chapter deals with the following; (1) the measurement method to assess the immunological level of individuals, (2) causes of immunological suppression, (3) possible methods of immunological restoration in elderly people.

2 Assessment of Immunological Level as a Whole

The immune system comprises various functions and consists of many types of cells that perform various functions, and it is difficult to select immunological parameters that are suitable for the assessment of immune functions in healthy people and patients suffering from various diseases.

From a functional viewpoint, there exist parameters such as cell mediated immunity, humoral immunity, cytokine production, proliferative activity of T-cell and B-cells, antigen presentation of dendritic cells and so on. Cells comprising the immune system are T-cells and their subpopulations, B-cells and their subpopulations, NK-cells, NKT-cells, macrophages and dendritic cells. None of these may be excluded for the assessment of the immunological level as a whole.

Another important aspect is to determine which immune cells or parameters play a key role in the age-related decline of immune function. We have been studying the immunological aspect of aging for many years and have confirmed that immune functions are susceptible to aging, diseases and stress. For the past 30 years, many studies including ours have shown that the age-related decline mainly occurs in T-cell-dependent immune functions and is relatively small in functions of other cells such as B-cells, macrophages and NK-cells (Makinodan and Kay 1980; Hirokawa 1992; Linton and Dorshkind 2004; Hirokawa et al. 2006). Therefore, when considering the age-related change of immunological functions, the immunological assessment can be focused on parameters that are related to T-cell-dependent functions. We have reported that the age-related change in the T-cell-dependent immune system is observed in a decrease in the T-cells number, a change in the T-cell subpopulations and a qualitative change in T-cells such as a decline in proliferative capacity and a change in cytokine production (Fig. 2).

Therefore, the number of whole T-cells and their subpopulations, and the proliferative activity of T-cells are useful parameters to assess the extent

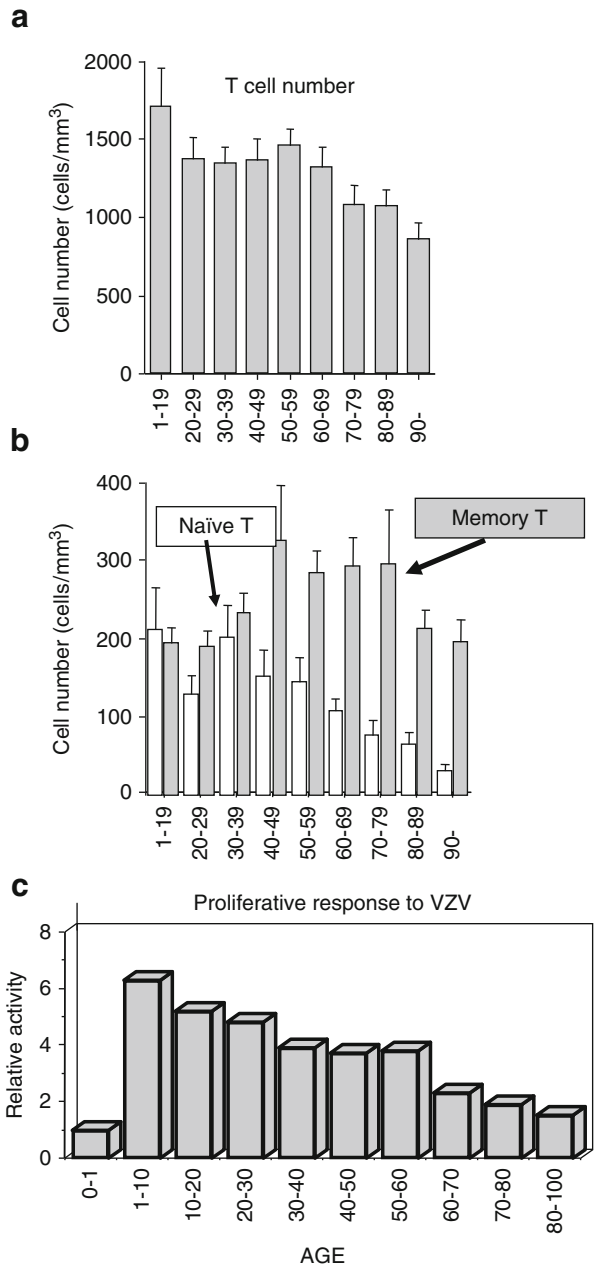

Fig. 2 Age-related decline in T-cell-related immune parameters
(a) A significant decline is observed between the 2nd and 3rd decades. The number stays at almost the same level through the 6th decade and declines after the 7th decade
(b) The age-related decrease in the number of naïve T-cells (open columns) with a concomitant increase in the number of memory T-cells (grey columns)
(c) Proliferative response of lymphocytes to varicella-zoster virus (VZV) peaks in the 1st decade and gradual decline thereafter. (The graph was originally made by Prof. S. Kishimoto)

of the age-related decline of immune functions. Hence, we performed flow cytometric analysis for 7 parameters reflecting T-cells and their subpopulations; number of T-cell ($CD3^+$ cells), number of $CD4^+$ cells, number of $CD8^+$ cells, the ratio of $CD4^+$ cells to $CD8^+$ cells (CD4/CD8 ratio), number of naïve T-cells

(CD4$^+$CD45RA$^+$ cells), number of memory T-cells (CD4$^+$CD45RO$^+$ cells), and the ratio of naïve T-cells to memory T-cells (N/M ratio). The proliferative activity of T-cells was measured by nonspecific stimulation of T-cells by anti-CD3 monoclonal antibody.

We defined a new indicator, T-cell proliferation index (TCPI) using the number and proliferative activity of T-cells, as described later.

In addition to T-cells, the number of B-cells and NK-cells was included for this assessment, since these cells are counterparts of immune functions.

Cytokine production is another important aspect of immunological function. Among many cytokines, information on Th1/Th2 balance is helpful for understanding health and disease condition. Therefore, we employed 3 interleukins; IL-2 and IFNg as the Th1 group, and IL-4 as the Th2 group.

Here, we have ten parameters to assess the extent of age-related decline in immunological functions (hereafter referred to as the scoring of immunological vigor: SIV) of individuals as shown in Fig. 3. Since it is difficult to imagine the immunological level of each individual by merely looking at the numbers of the 10 parameters, we have tried to represent the immunological level of each individual

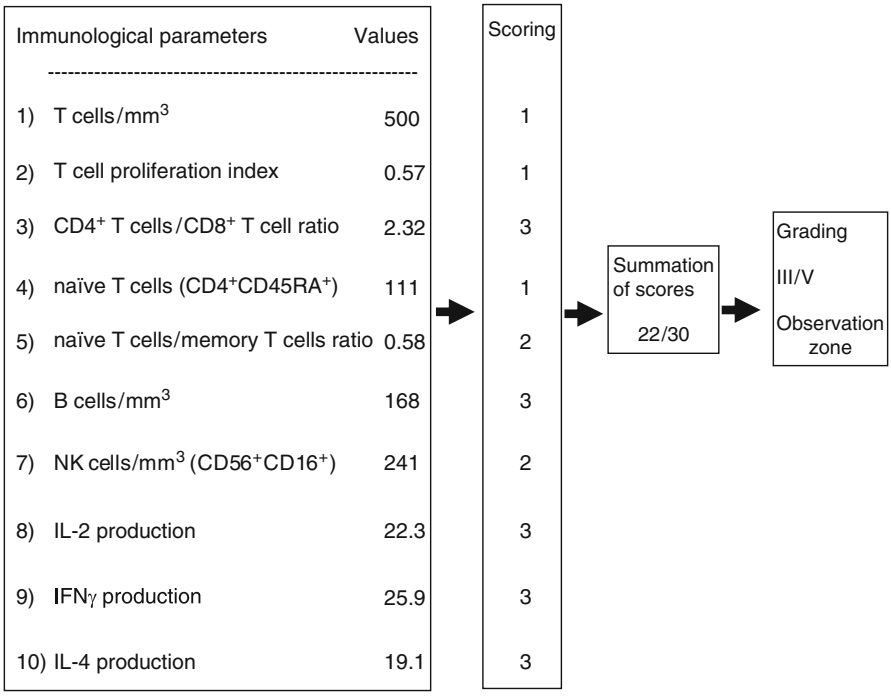

Fig. 3 Process of scoring and grading immunological parameters. Values of immunological parameters are given a score from 1 to 3. Sum total of 10 scores is named scoring of immunological vigor (SIV-10). SIV-10 is then classified into 5 grades as shown in Table 2. It is generally difficult to determine immunological status of individuals by a mere list of figures of 10 parameters. By the scoring system, 10 parameters can be grouped and expressed numerically as immunological score or grade

in a plain style. The value of each immunological parameter falls within a range specified in a database and each parameter was scored into 3 grades based on its value. In particular, values in the range of a cumulative frequency less than 10% of values observed for healthy subject were scored 1, which indicates a low immunity level; those between 10% and 40% were scored 2, which indicates a moderate immunity level; and those with 40% or higher were scored 3, which indicates a sufficiently high immunity level. Since higher scores of CD4/CD8 ratios are frequently observed in very old people and patients suffering from diseases, values greater than 80% of the cumulative frequency were scored 2, which indicates a moderate immunity level.

We employed 3 options to assess SIV; the first using 10 parameters, named SIV-10; the second using 7 parameters excluding cytokine production, named SIV-7 and the third using 5 parameters composed of T-cell-related functions, named SIV-5 or T-cell immune score.

Values of SIV-10, SIV-7 and SIV-5 were then classified to 5 grades (V~I) according to the total score. V: sufficiently high, IV: safety zone, III: observation zone, II: warning zone, I: critical zone (Table 2). The observation zone indicates that the level of SIV is average, but needs attention to move up into the safety zone. The warning zone indicates that the level of SIV is not sufficient to maintain health and considerable effort is required to increase the level of SIV. The critical zone indicates that the susceptibility to infection is so high that the individual needs to be admitted to an aseptic isolator.

3 T-cell Proliferation Index and Assessment of Immunological Age

We defined a new parameter, T-cell proliferation index (TCPI) which was calculated by using the number and proliferation activity of T-cells. The number of T-cells is essential for the maintenance of immune function. In addition, T-cell proliferation is the most essential function of T-cell immunity, including the process of antigen recognition and sequential division of T-cells. There are 4 types of number and proliferative capacity of T-cells. (1) both are sufficient; (2) number is sufficient,

Table 2 Scoring of immunological vigor (SIV) and grading

Scoring			
SIV-10	SIV-7	SIV-5	
10 parameters	7-parameters (without cytokines)	5parameters (T-cell related)	Grading
30 ~ 29	21	15	Grade V sufficiently high
28 ~ 26	20 ~ 18	14 ~ 13	Grade IV safety zone
25 ~ 22	17 ~ 13	12 ~ 10	Grade III observation zone
21 ~ 17	12 ~ 10	9~ 7	Grade II warning zone
16 ~ 10	9 ~ 7	6 ~ 5	Grade I critical zone

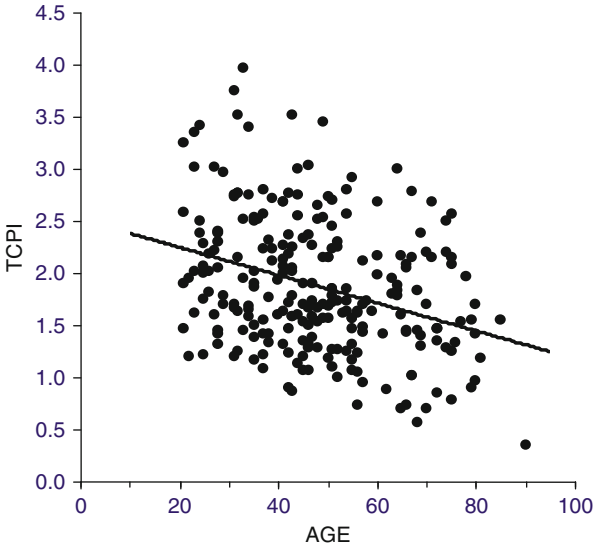

Fig. 4 Distribution of TCPI against age. Approximate values of 400 people are displayed

but proliferative capacity is insufficient; (3) number is insufficient and proliferative capacity is sufficient; (4) both are insufficient. Therefore, we arrived at the conclusion that we needed a new parameter which reflected both the number and proliferative capacity of T-cells. The new parameter was TCPI and was calculated by the following equation.

TCPI = T-cell proliferation activity x (T-cell number per μL/1000)

Figure. 4 indicates the distribution of TCPI according to age. Although, there is a wide individual variation, the age-related decline of TCPI is obvious and the following equation was obtained.

TCPI = – 0.0174 x (Age) + 2.5348

In other words, we can determine age of individual by the value of TCPI using the following equation. Since the age is calculated from the number of T-cells and their proliferative activity, the age obtained by this equation is hereafter referred to as the immunological age (IA).

IA = (2.5348 – TCPI)/0.0174

4 Scoring of Immunological Vigor (SIV) Shows Wide Range of Individual Variation

Immunological vigor of 400 healthy people and 300 cancer patients were assessed using the method mentioned above (Fig. 5a). Age-related related decline as observed in SIV-7 was apparent in the healthy population. However, there was a wide individual

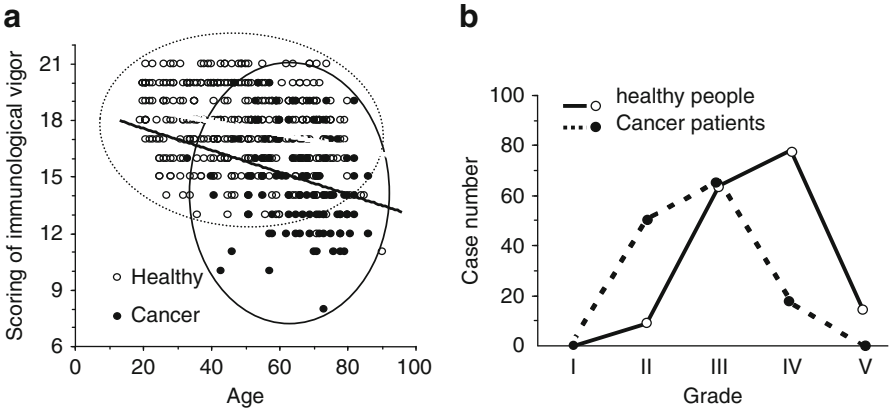

Fig. 5 Comparison of SIV-10 (a) and grade (b) between healthy people (400 cases) and cancer patients (300 cases)

variation. The level of SIV-7 ranged between 21 and 14 in the 3rd decade and between 21 and 12 in the 7th decade, indicating that individual variation is greater than the difference between young and old people. But when observing SIV-7 in cancer patients, we found that the distribution of values was apparently different from that in healthy people and the age-related decline in cancer patients was much steeper than in healthy people. However, it is again apparent that the SIV-7 level of cancer patients showed a wide range of individual variation, although the values were lower as compared with those for healthy people (Hirokawa et al. 2007a, 2007b).

Based on the SIV-7 grade, the difference in SIV-7 between healthy people and cancer patients was much obvious (Fig. 5b). Most healthy people belonged to Grade IV and III, while cancer patients belonged to Grade III and II, indicating that the immunological deficient state is more serious in cancer patients. Thus, considerable attention should be given to the immunological state of cancer patients during treatment (Hirokawa et al. 2008).

Figure. 6 shows radar graphs of 3 example cases showing the 10 immunological parameters, IA, SIG-10, immunological grade and immunological zone. This figure shows how the immune status of individuals can be understood.

5 Variable Causes Suppress Immunological Functions

As presumed from the wide individual variation of immunological vigor, immunological functions are changeable by variable causes: i.e., aging, stress, diseases, life style, food, genetic background etc.

Aging is unavoidable cause of immunological decline. However, the rate of immunological decline by aging might be accelerated or decelerated by surrounding environmental factors which can be controlled by appropriate intervention. For

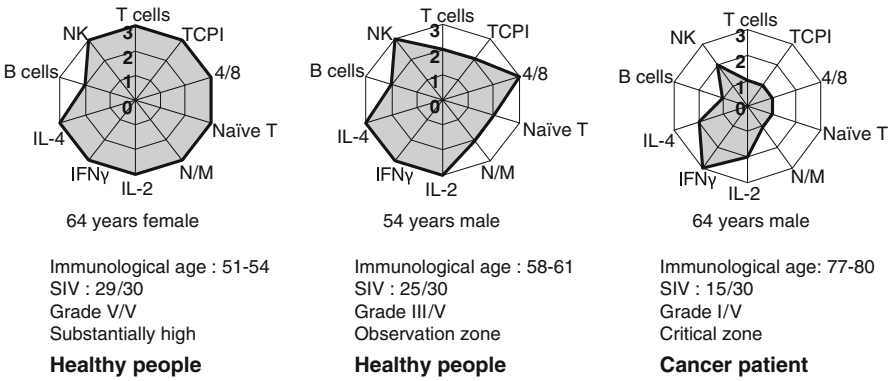

Fig. 6 Radar graph shows 3 examples assessed by immunological age, SIV and grade

instance, caloric restriction extended the life span of animal models and enhanced the immunological function of old animals (Heilbronn and Ravussin 2003; Utsuyama et al. 1996).

Stress is the major cause of immunological decline. Any forces that disturb homeostasis can be stressors. In other words, stress is life and life is stress (Chrousos GP et al. eds; Stress 1995).

Various diseases including minor ones are also factors suppressing immunological functions. Incidence of cancer is known to increase with the advancement of age and this is partly caused by a decrease in immune surveillance (Dunn et al. 2002). In fact, a patient with colonic cancer has a low level of SIV (Hirokawa et al. 2008). But it is obvious that SIV generally recovers in a certain interval after the removal of cancer lesion. Thus, it is likely that the presence of cancer suppress the immunological function of the individual. It is also well known that diabetes mellitus suppresses immunological function. Various therapies for many kinds of diseases can down-regulate immunological functions. One example is surgical operation. Chemotherapy for cancer and steroid therapy for autoimmune diseases suppress immune functions.

6 Possible Methods of Immunological Restoration

Now we can assess the immunological level by SIV, as mentioned above. As observed in Fig. 5, there is a wide individual variation in the SIV levels. We presume that individuals with high SIV can live longer and those with low SIV are susceptible to diseases. Further longitudinal studies are necessary to say something for the prognosis of people with high or low SIV. Roberts-Thomson et al. reported that individuals with reduced immune functions had significantly greater mortality than those whose immune functions were within the normal range. Centenarians in Okinawa have significantly high immune functions as compared with controls. Considering these data, it may be inferred that it is desirable to enhance SIV level of individuals with a lower SIV level.

Here we would like to present our preliminary experience of immunological restoration in humans and its possible methods as determined from experiments using animal models (Hirokawa et al. 2002).

6.1 Coping with Stress

In daily life, a variety of stresses down-regulate immune functions and in fact, the stresses are the major cause of immunological suppression in our life. Stresses originate from communication problems at home, office and school, from diseases or injury, from treatment of diseases or from natural disasters. There are 2 solutions for stress: (a) avoid stress. (b) alleviate the effect of stress.

(a) Avoid stress.

Needless to say, it is best to avoid stress if possible. Unfortunately, however, most stresses are unavoidable. But it is worthwhile to reconsider the cause of stress and analyze whether or not it is avoidable.

(b) Alleviate stress.

It is interesting to note that the same level of stress has very serious effects in some people, but not in others. This means that the magnitude of stress is dependent on the response of individuals. For instances, an academic examination would not be as serious for students who have studied beforehand as it would be for those who have not studied.

Singing, running and chattering with friends are mood-altering activities. Sleeping, if possible, is another good activity. A good way to alleviate stress is to devote time to some hobby. In this respect, we studied the effect of music in humans and we found that playing the drums in a group could enhance NK activity (Wachi et al. 2006).

6.2 Improvement of Life Style

Habits indicative of an improper life style are poor sleep, overwork, smoking, excessive drinking, irregular meals, deviated food habits and insufficient physical exercise. We interviewed more than 50 of young people who showed a lower level of SIV and over 50% of them had improper work habits.

Nutrition is an important factor for immunological restoration. For the last 50 years, the mean life span of Japanese people has extended from 50 to over 80 years. This rapid increase in life span is partly due to the improvement of food intake, especially, protein. Today, people are worried about metabolic syndromes and are making an effort to not eat too much. But too much of anything is harmful.

More than 70 years ago, MaCay (1935) reported the elongation of life span in rats by caloric restriction. Many studies have since shown that caloric restriction is effective on several counts; i.e., elongation of lifespan, enhancement of immunological function and alleviation of autoimmune diseases.

One argument about food restriction or caloric restriction is that it only appears to be effective in animals that are reared in an artificial environment such as a laboratory animal colony (Utsuyama et al.1996). Most laboratory animals are fed highly nutritional chow without being given enough space or tools for physical exercise. In other words, immunological improvement by food restriction might be seen only in overfed animals that do not perform physical exercise. In order to test this conjecture, we employed an automatic feeding device that was electrically interlocked with a running wheel: the device provided constant running exercise to the animal and fed a determined amount of diet to each rat (lchikawa et a1. 2000).

Rats were individually reared in this automatic feeding device for 18 months from 2 to 20 months of age. At the age of 20 months, the rats were sacrificed and examined for various indices including immunological functions. The body weight was almost the same between rats fed ad libitum and those given 80%-restricted diet. Their body weight was significantly greater than that of rats given 60%-restricted diet. The body weight of those given 80%-restricted diet together with physical exercise was almost the same as that of rats given 60%-restricted diet without physical exercise. High proliferative response of T-cells was observed in some of rats given 80%-restricted diet together with physical exercise (Fig. 7).

Meanwhile, studies are ongoing to observe the effect of caloric restriction on various physiological parameters in monkeys (Roth et al. 2002). A report provided evidence that CR can delay immune senescence in nonhuman primates, potentially contributing to an extended lifespan by reducing susceptibility to infectious disease (Messaoudi et al. 2006).

In humans, the effects of CR on life extension are actually present; it is estimated that CR extends life by 3–13 years. This extension is much smaller than those achieved by medical and public health intervention, which have been known to extend life by about 30 years in developed countries of the 20th century (Everitt and Le Couteur, 2007).

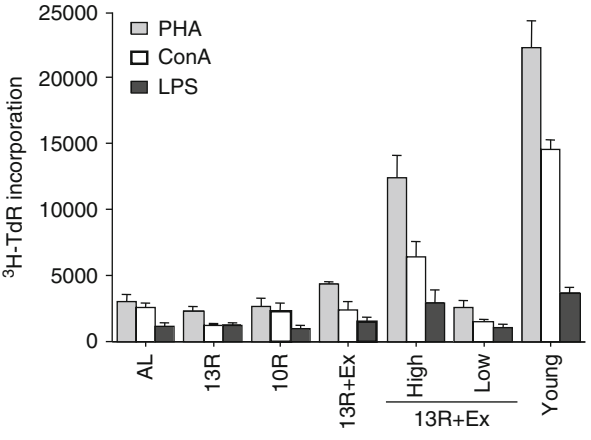

Fig. 7 Effect of long-term exercise and caloric restriction on lymphocyte proliferation in rats

6.3 Anti-oxidant Chemicals

It is now commonly believed that oxidative stress, such as that caused by reactive oxygen species and free radicals, is the major cause of aging phenomena and various diseases (Harman 1956; Ames et al. 1993; Wakikawa et al. 1999). The decline of immune function is one of major aging phenomena and constitutes the background of various diseases occurring in the elderly people (Hirokawa 1998). Many investigators assume that oxidative stresses play an important role in the progression of immunological decline during the course of aging (Martin et al. 1996; Meydani et al. 1998). Thus, it has been expected that enzymes or substances that compete with oxidative stress are candidates for antiaging medicines. Anti-oxidant enzymes are present in our body, e.g., SOD and catalase. It is possible to enhance the production of these antioxidant enzymes by appropriate physical exercise. In addition, there are many kinds of supplements that are commercially available as antioxidant chemicals, although effectiveness of most of them has not been proved scientifically.

Flavonoids or polyphenols are representative antioxidants contained in many kinds of vegetables, fruits and other foods. Extracted or enriched flavonoids and polyphenols are commercially available as supplements. The effectiveness of these supplements requires further study.

Vitamin E (VE) works as an antioxidants. Research groups have found conflicting effects of VE. In our experiment using aging mice, we confirmed that VE enhances the immune functions of young, but not old mice (Wakikawa et al. 1999) (Fig. 8). Other reports indicated that VE supplementation was not effective in humoral immune response modulation in young, middle-aged and elderly women (Park et al. 2003). On the other hand, Meydani's group reported the positive effect of VE in both human and animal experiments (Meydani et al. 1998; Marko et al. 2007). These reports suggest that the effectiveness of supplements is not uniform, but differs with the individual and the genetic background.

The effectiveness of any antioxidants and related supplements usually varies with the individual. Therefore, immunological assessment as performed in an objective

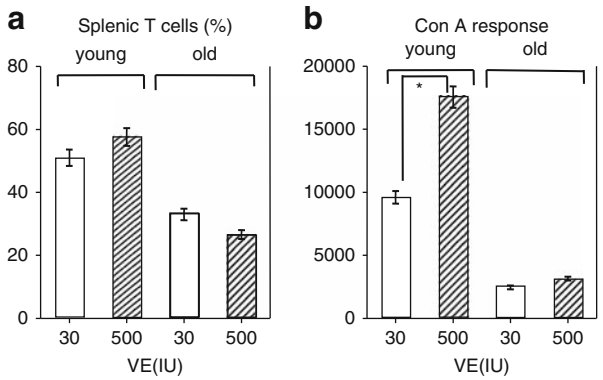

Fig. 8 Effect of vitamin E on splenic T-cells (a) and Con A response (b) of young and old mice

manner, such as determining SIV, is always necessary to verify the effectiveness of chemicals and supplements on the immune system.

6.4 Vaccine

Vaccines are very useful to protect children who are susceptible to various infectious diseases due to immaturity of the immune system. The same effect could be expected for the elderly people with impaired immune functions. However, vaccination in very old people may not work due to depressed immune functions. Thus, vaccination should be started latest before the age of 60, so that the individual still have sufficient capacity to respond to pathogenic organisms. Alternatively, attempts should be made to enhance or stimulate the depressed immune capacity of elderly people at the time of vaccination.

Susceptibility of old mice to infection is clearly observed in experimental infection with influenza virus. Old mice died of infection at a 10-fold lesser dose of influenza virus. 01d mice that survived after exposure to low doses showed strong immunity to the same virus and became resistant to the second infection with a high dose of influenza virus (Hirokawa and Utsuyama 2002). These data suggest that vaccination is useful to protect the elderly people from various kinds of infection.

In humans, vaccination against pneumococcus and influenza virus is already clinically conducted for elderly people. As stated in the earlier section, there is individual variation even in elderly people. Those having higher SIV can respond to vaccination and show sufficient immunity against bacteria and virus. Problem is in finding a way to enhance immunological functions of the elderly people with lower SIV so that they can effectively respond to vaccination.

Another point to be considered is the route of vaccination. Since the nose and mouth are the major entries for respiratory infection, their mucosal immunity is important. Using a mouse model, we found that the route of vaccination is important. Vaccination through the intranasal route provides high level of IgA production in nasal mucosa, but that through the intravenous and intraperitoneal routes does not. (Table 3). (Asanuma et al. 2001).

Table 3 Difference in the levels of IgA antibody specific to influenza virus (ng/mouse) between vaccination route and age

	3 months old	18 months old
1) i.n. → i.n.	172 ± 42	54 ± 50
2) i.v. → i.p.	2 ± 2	2 ± 2

1) Mice were administered intranasally (i.n.) with A/PR/8/34 vaccine (10mg) and boosted 3 weeks later i.n. with the same vaccine. Antibody was assessed 1 week after the boosting

2) Mice were administered intravenously (i.v.) with A/PR/8/34 vaccine (10mg) and boosted 3 weeks later intraperitoneally (i.p.) with the same vaccine. Antibody was assessed 1 week after the boosting

6.5 *Japanese Herbal Medicines*

A group of Japanese herbal medicines, called 'Kampo-Hozai' have been used to improve the physical condition of patients suffering from various diseases (Utsuyama et al. 2001). Among more than 100 kinds of Kampo-Hozais, Hochuekki-to(TJ-41) is a drug used to recover immune function and has been reported to be useful in healing infections (Yamaoka et al. 2000; Mori et a1. 1999), oncostatics-induced leukopenia (Kaneko et al. 1999), and allergies (Suzuki et a1. 1999). Juzen-taiho-to (TJ-48) is useful not only for the recovery of immune function (Abe et a1. 1998), but also for the enhancement of antitumor effects (Saiki et al. 1999; Onishi et al. 1998; Utsuyama et al. 2001).

We tested the effectiveness of Japanese herbal medicines in young and aged mice. The data indicated that Hochu-ekki-to (TJ-41) was effective in the restoration of impaired immune functions of aged mice, in terms of the number of T-cells and NK-cells, and anti-SRBC antibody response. However, it was not effective in enhancing the immune functions of young mice (Fig. 9).

Juzen-taiho-to was also effective in increasing the number of T-cells and NK-cells in aged mice, although a significant increase was not observed in young mice. Functionally, however, NK activity increased both in young and old mice. A significant decrease was also observed in the number of metastatic pulmonary colonies of B16 melanoma cells both in young and old mice treated with Juzen-taiho-to for 16 weeks (Fig. 10).

These results suggested that some Japanese herbal medicines are useful in the restoration of impaired immune functions of old mice and could be recommended for the elderly people with immunological problems.

We performed preliminary clinical trial of TJ-41 to observe its effect on immunological function in humans. The results showed that the effect varied with the individual. Approximately half of the people who were given TJ41 showed a positive effect, but another half did not show any effect. Fig. 11 shows an example case of the positive effect of TJ41. Therefore, in these cases also, the assessment of immunological parameters is necessary to verify the effectiveness of herbal medicines for immunological restoration.

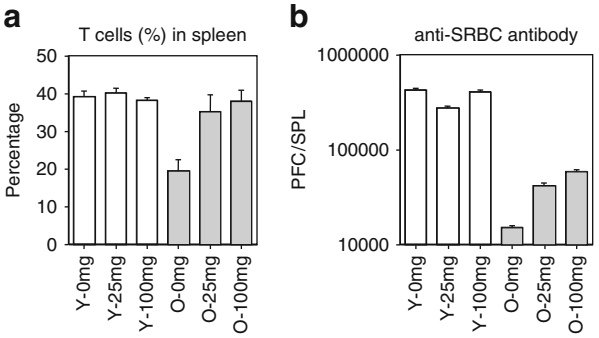

Fig. 9 Effect of Japanese herbal medicine (TJ-41) on splenic T-cells (a) and anti-SRBC response in young and old mice

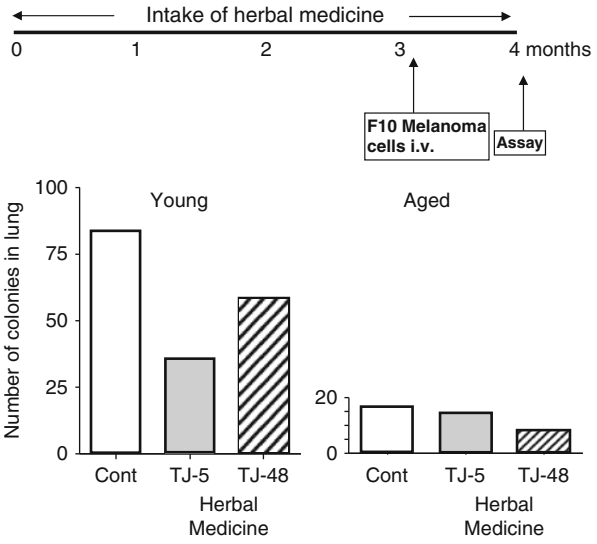

Fig. 10 Effect of Japanese herbal medicine (TJ-48) on tumor metastases to lungs in young and old mice. Top figure shows experimental protocol. TJ-5 is another Japanese herbal medicine, Anchusan

6.6 Hormones

After gonadectomy, hypertrophy of the thymus was observed in aging C57BL/6 mice, ranging in age from 4 to 20 months. The mice had been gonadectomized 1 month before the sacrifice, and the magnitude of thymic regeneration was more pronounced in males than in females (Fig. 12). However, enhancement of anti-SRBC antibody response was observed only in females, but not in males regardless of age. Gonadectomy brought about not only thymic hypertrophy but also an increase in T-cells and B-cells in the spleen. An increase in T-cell subpopulations was proportional in female mice, but disproportional in male. The disproportional increase of T-cell subpopulations could account for the failure to enhance the anti-SRBC antibody response in male mice (Utsuyama and Hirokawa 1989).

Gonadectomy also resulted in the thymic hypertrophy in male and female young Wistar rats, but not in those that had been previously hypophysectomized (Utsuyama and Hirokawa 1989).

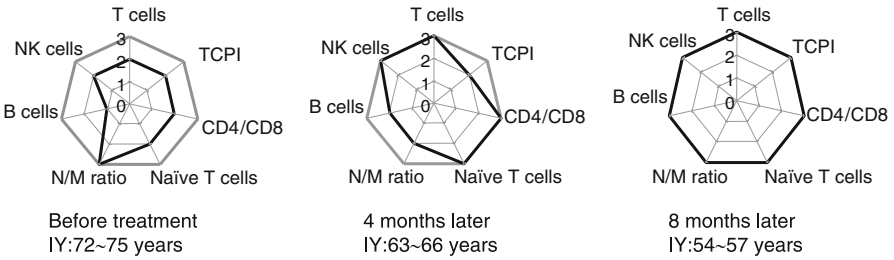

Fig. 11 Effect of TJ-41 on immunological parameters and immunological year (IY) in 66 years old male. Full recovery was observed 8 months later

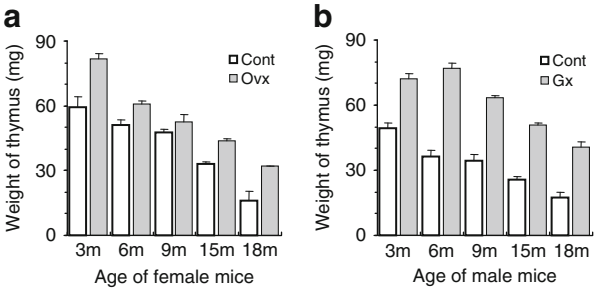

Fig. 12 Effect of ovariectomy (a) and orchidectomy (b) on the weight of the thymus at various ages. All mice were sacrificed 1 month after the gonadectomy and the wet weight of the thymus was measured

Elderly males undergoing orchidectomy for prostatic carcinoma demonstrated an increase in circulating T-cell numbers, particularly naive (TREC+) T-cells. Chemical castration by administration of LHRH antagonist was also effective in activation of thymic regeneration in mice and humans (Sutherland et al. 2005).

In females, hormone replacement therapy (HRT) by estrogen has been prescribed to postmenopausal women for prevention of a variety of medical conditions including osteoporosis, cardiovascular diseases, stroke and Alzheimer's disease; yet HRT is often associated with altered immune parameters (Fahlman et al. 2000; Stopinska-Gluszak et al. 2006). HRT is now going to be reconsidered, since estrogen is closely related with carcinogenesis of mammary cancer.

Growth hormone plays a key role in the development and aging of the thymus and T-cell-dependent immune system (Hirokawa et al. 1998, 2001). Activation of the immune functions occurred in both males and females by administration of GHRH (Khorram et al. 1997; Koo et al. 2001). Since elderly people frequently have occult carcinoma in thyroid, prostate and other organs, we need to consider the possibility that an increased level of growth hormone may stimulate proliferation of tumor cells, giving rise to the clinical manifestation of the occult carcinoma (Perry JK 2006).

6.7 Immunological Enhancement by a Low Dose of Anti-cancer Drug

Cyclophosphamide (CY) is an antitumor drugs commonly used for the chemotherapy of human cancer. It is also known to be a potent immunosuppressive drug in human and experimental animals. It is interesting how CY influences the impaired immunological function in aged mice (Ishiyama et al. 1999).

Aged mice treated with a low dose of CY showed significantly enhanced immune capacity in terms of T-cell proliferation and T-cell-dependent antibody response (Fig. 13). In these mice, the total cell numbers of T-cells increased in both in the thymus and spleen, as compared with those in nontreated mice. Treatment with a low dose of CY induced apoptosis of thymocytes in the atrophic thymus of aged mice and this was followed by an increase in proliferation of thymocytes and an increase in thymocytes and splenic T-cells. Treatment with a high dose of CY also

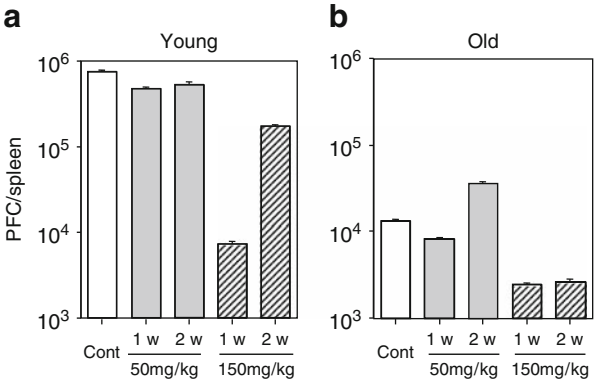

Fig. 13 Restoration of anti-SRBC response of old mice by administration of low dose (50mg) of cyclophosphamide, but not by a high dose (150mg). In young mice, the suppressive effect was obvious

induced apoptosis in the thymus, but suppressed the proliferative capacity, thereby, not leading to an enhancement of immune capacity.

CY in young mice, however, suppressed immune capacity regardless of the dose. Thymocytes and splenic T-cells of young mice were more susceptible to CY than those of aged mice and decreased in number after treatment with even a low dose of CY.

For human application, Berd et al. (1984) reported that a low dose of CY enhanced cell-mediated and humoral immunity in patients with advanced cancer. We examined SIV in patients with colorectal cancer before and after chemotherapy and found that 8 out of 13 patients showed a trend of increased SIV 8 weeks after the start of chemotherapy (Fig. 14).

6.8 Grafting of Cells and Tissues

In animal experiments, the level of immune functions of aged mice can be restored to a level approaching that of young adult mice by grafting both newborn thymus and bone-marrow from young donors (Hirokawa et al. 1976, 1982; Hirokawa and Utsuyama 1989). The results suggest that intrinsic cellular change of the immune system is more responsible for the immune deficiencies in the aged than the environmental or structural tissue changes including connective tissues and humoral factors. In thymus grafting, however, thymic stromal tissues rather than thymocytes are important for the restoration of T-cell-dependent immune system. For human application, the transplantation of bone-marrow cells is becoming easier, but it is almost impossible to find donors of young thymuses. Further, donors need to have the same MHC type as the recipients. Thymus transplantation may be performed, if an autologous thymus can be partially removed at a young age and stored in liquid nitrogen.

Meanwhile, researchers have been accumulating data on molecules of thymic epithelial cells that are essential for T-cell differentiation (Zuniga-Pfucker 2004; Utsuyama et al. 2003). Accordingly, in the near future it may be possible to reconstruct an artificial thymus using easily available cells of individuals.

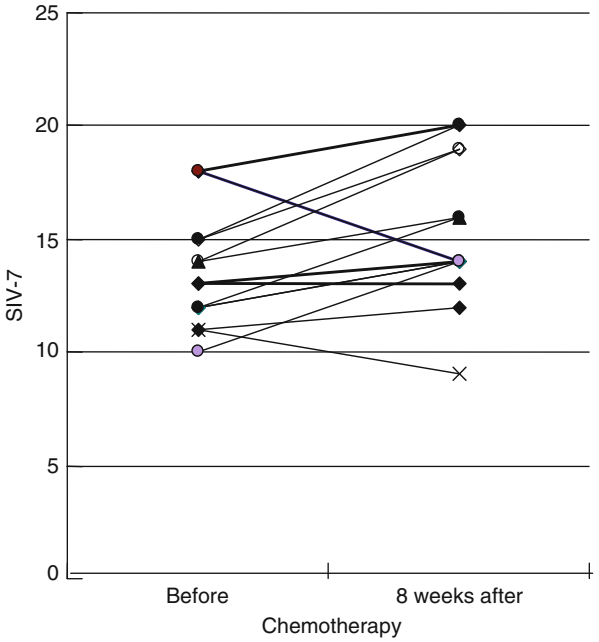

Fig. 14 Patients with colorectal cancer, (13 cases), treated by chemotherapy. Eleven out of 13 cases showed a trend of increase in SIV-7, 8 weeks after the onset of chemotherapy

6.9 Infusion of Activated Autologous T-cells

Cell transfer method using young and old mice showed that 10% of the age-related decline can be attributed to cellular environment and 90% to changes intrinsic to the old cells (Price and Makinodan 1972). Many studies including ours (Hirokawa et al. 2006) have revealed that the intrinsic cellular changes are mainly observed in T-cells; i.e., decrease in number, changes in composition of subpopulations and qualitative changes such as proliferative activity and cytokine production.

In fact, T-cells from old individuals do not proliferate efficiently in vitro, but the proliferation can be promoted in the presence of anti-CD3 and IL-2. Thus, activated T-cells in vitro are expected to restore the declined immune functions of aged mice and humans. This treatment was already employed for cancer treatment as an immunotherapy, e.g., lymphokine activated killer cells (LAK) (Rosenberg 2001).

6.9.1 Animal Models

We tested the effect of infusing activated T-cells using young and old mice (Hirokawa et al. 2007). In this study we employed a congenic combination of B10.Thy1.1 mice (young and old) as donors and C57BL/6 Thy1.2 mice (young and old) as recipients, to determine how many activated T-cells survived in the recipients. The mice were sacrificed 11 days and 25 days after the infusion of activated T-cells and used for immunological assessment. For the infusion of activated T-cells, splenic lymphocytes were expanded 10- to 15-folds in the presence

of immobilized anti-CD3 monoclonal antibody and IL-2. Lymphocytes activated in this way were composed of mostly T-cells in which approximately 70 to 80% were $CD8^+$ T-cells and 7 to 14% were $CD4^+$ T-cells. The activated T-cells prepared from old mice donors contained many more CD8 T-cells. Although CD4+ T-cells were smaller in number than CD8+ T-cells, most of them expressed a phenotype of naïve T-cells. After infusion of activated T-cells, the absolute number of T-cells significantly increased in the spleen of the recipient mice, especially of old mice. In the peripheral blood and spleen, donor-type Thy-1.1 T-cells were significantly more numerous in old recipients than in young ones. In addition, the number of donor-type T-cells that survived was significantly high in the spleen than in the peripheral blood in both young and old recipients.

The magnitude of antibody formation against SRBC did not change significantly in young recipients. About half of the old recipients, however, showed a significant enhancement of antibody formation. It is of importance to note that such an enhanced antibody formation was observed in old recipients infused with activated T-cells either from young or old donors.

6.9.2 Trials in Human Cancer Patients

T-cells from peripheral blood of healthy people can be easily expanded more than 1000-fold in vitro in the presence of immobilized anti-CD3 monoclonal antibody (MoAb) and IL-2. The infusion of activated autologous T-cells has been widely used for cancer patients as a form of immunotherapy (Rosenberg 2001), but without significant impact on cancer treatment in many cases. However, it can be expected that activated T-cells expanded in vitro in a nonspecific manner may improve the immune deficient status of elderly people and cancer patients. T-cells expanded in a nonspecific manner may contain harmful T-cells exhibiting autoimmune activity. In this respect, a recent paper reported that the infusion of activated autologous T-cells did not enhance or promote autoimmune activity (Yamaguchi et al. 2004).

In the next step, we examined cancer patients in the advanced stage; patients with tongue cancer (1 case), esophageal cancer (2 cases), lung cancer (4 cases), gastric cancer, pancreatic cancer (3 cases), colon cancer, appendical cancer and ovarian cancer. All the patients were in the advanced stages of cancer, with multiple metastases, and they underwent an infusion of autologous activated T-cells (so-called LAK cells).

The activated autologous T-cells prepared in the study comprised T-cells (99%), and the CD4/CD8 ratio was approximately 2–3. The proportion of NK-cells was less than 1% in the activated T-cells.

The number of cell per infusion was approximately 5×10^9, and the infusion was repeated 5–6 times for 10 weeks. Various parameters were examined before and after the infusion of activated autologous T-cells.

Figure15 shows the immunological parameters before and after the infusion of activated autologous T-cells in an advanced cancer patient. Most of the immunological parameters that were examined improved after the infusion, except for the ratio of T-cell subpopulations.

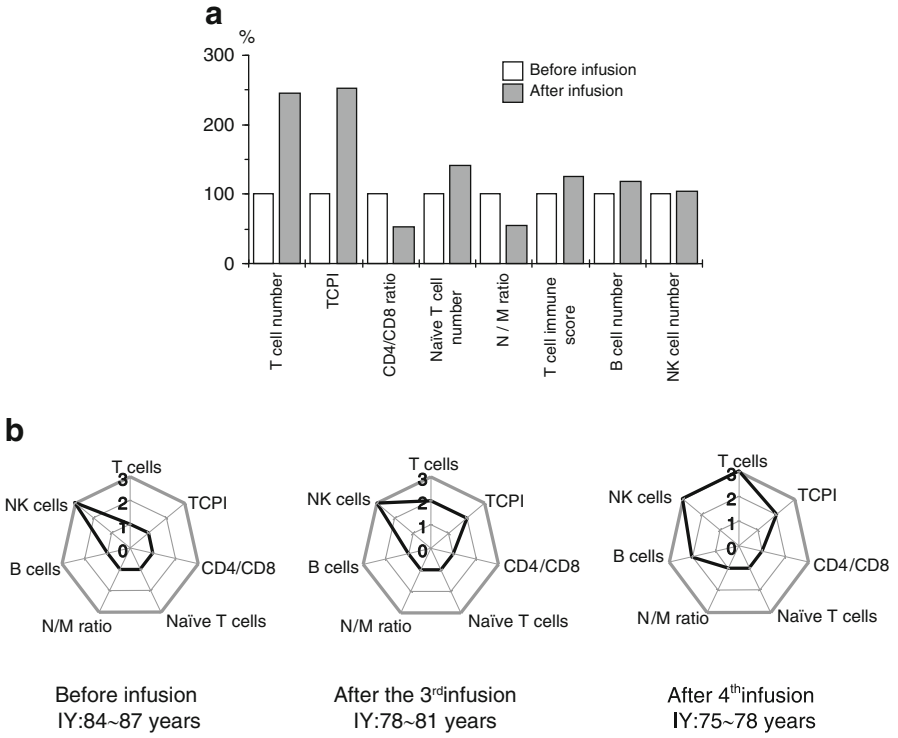

Fig. 15 Two cases of cancer patients, treated with infusion of activated autologous T-cells (a) Improvement of immunological parameters was observed in 60-years-old male with lung cancer. Improvement was observed in most parameters except for the ratios of T-cell subpopulations such as the CD4/CD8 ratio and N/M ratio (b) Infusion of activated autologous T-cells gradually improved radar graph patterns and immunological year (IY) in 78-years-old female with lung cancer

We examined the effect of the infusion of activated autologous T-cells in 14 cases and found that the most pronounced improvement was observed in the T-cell proliferation index (TCPI). The values varied across cases and were unsuitable for statistical analysis. After the infusion of activated autologous T-cells, an improvement in TCPI was observed in 11 out of 14 cases. The average value of TCPI before the infusion was 1.02, which is apparently lower than that observed in colonic cancer stages I to IV (1.21). After the infusion, the average TCPI was increased to 1.49, although it was definitely lower than that of healthy controls (1.70). It was not obvious whether the infusion of activated autologous T-cells was effective in reducing the tumor size, but most of the patients revealed that in general, they experienced an improvement in their health status after the infusion.

Improvement of immunological function can not be expected in cancer patients whose T-cell expansion in vitro is less efficient. Thus, we have to consider the source of T-cells. A good technique is to obtain T-cells not from patients suffering from cancer, but from individuals in healthy condition. For this purpose we need to obtain peripheral blood lymphocytes from healthy peoples and these lymphocytes should be kept in the frozen state. The process can be named as T-cell-bank and the system to establish T-cells bank is now under way.

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Thymic Regeneration in Mice and Humans Following Sex Steroid Ablation

Anne Fletcher, Jessica Reiseger, Katerina Vlahos, Natalie Seach, Jarrod Dudakov, Ann Chidgey and Richard Boyd

Abstract: The thymus is the cradle of T-cell-mediated immunity. Normal thymic development ensures the export of a diverse repertoire of T-cells reactive against pathogens, foreign matter and tumours, and its role as the sole generator of αβT-cells makes it a unique organ, indispensable for health.

Although the thymus continues to produce T-cells throughout life, it undergoes progressive atrophy with age, restricting both the number and diversity of newly derived T-cells within the peripheral T-cell pool and compromising their ability to

R. Boyd(✉) · J. Reiseger · K. Vlahos · N. Seach · J. Dudakov · A. Chidgey · A. Fletcher
Monash Immunology and Stem Cell Laboratories
Monash University
Clayton, Australia
Tel: +61 3 9905 0630
Fax: +61 3 9905 0690
E-mail: Richard.Boyd@med.monash.edu.au

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detect and respond efficiently to pathogens. This involution is most obvious from the onset of puberty and, accordingly, can be reversed with sex steroid ablation (SSA) therapy. Clinically, this is of paramount importance for patients with acquired immunodeficiencies, since the atrophied thymus cannot quickly export sufficient numbers of new T-cells to repopulate a depleted peripheral pool. Instead, patients are left dangerously susceptible to infection for extended periods. Reversible SSA promises to speed the time taken to immune recovery by rejuvenating the aged thymus and increasing T-cell output, with the potential to transform the clinical management of many major diseases with T-cell based aetiology.

Keywords: Thymus • T-cells • regeneration • Sex steroids • Bone marrow transplantation

1 The Thymus

The thymus is mainly composed of haemopoietic-derived tissue, including developing T-cells, antigen-presenting dendritic cells (DC) and phagocytic macrophages. The nonhaemopoietic thymic stromal compartment of epithelial and connective tissue comprises about 0.5% of total thymic cellularity and forms a framework or niche through which thymocytes migrate, interact and develop before export to the periphery as mature, self-tolerant T-cells (Boyd et al. 1991; Anderson et al. 2001; Schmitt et al. 2005; Takahama, 2006).

Each lobe of the thymus contains continuous lobules, further delineated into 5 zones: subcapsule, cortex, cortico-medullary junction, medulla and perivascular space (Fig. 1). (Godfrey et al. 1990; van Vliet et al. 1984; Boyd et al. 1991; Anderson et al. 2001). Far from a static support, distinct stromal subpopulations provide a unique combination of direct ligand/receptor interactions, chemokines, cytokines and growth factors, fundamental to the migration, differentiation and development of the thymocytes. Mature, antigen-naïve thymocytes that are exported to the periphery have been selected by thymic stromal cells and dendritic cells for their ability to bind self-MHC, and their low reactivity for self-peptide (for reviews, see Page et al. 1996; Anderson et al. 2001; Gatzka et al. 2007).

1.1 Classical αβ T-cell Development

During thymopoiesis, haemopoietic precursors sequentially acquire and lose distinct surface markers, undergo somatic rearrangement of their TCR genes in the shaping of their TCR repertoire, and gain specific T-cell functions (Fig. 1). Throughout this process of differentiation via distinct stages of development, thymocytes migrate between microniches in a directed fashion, assisted by chemokines produced by specific stromal cell subsets.

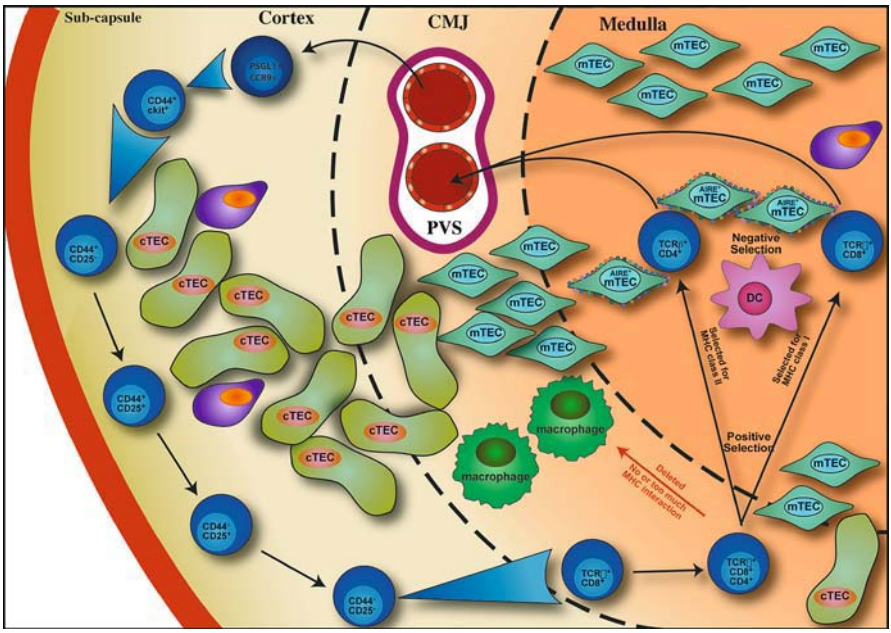

Fig. 1 T-cell development involves interaction with characteristic stromal cells in various microniches

Bone-marrow derived T-cell progenitors enter the thymus via endothelium at the cortico-medullary junction (CMJ); a carefully-regulated process involving interactions with CD44, α4 and β2 integrins, P-selectin glycoprotein ligand-1 (PSGL1) and the chemokine CCL9 (Lesley et al. 1985; Wu et al. 1993; Rossi et al. 2005; Schwarz et al. 2007). DN1 cells are CD44+CD25- and lose expression of ckit in early developmental events before upregulating CD25 (DN2) as they migrate through the cortex. Cells undergo positive selection at the DN3 stage (CD44-CD25+) through interactions with MHC expressed by cTEC, which, if successful, induce downregulation of CD25 (DN4) followed by proliferation and expression of CD8 and CD4 (DP). DP thymocytes express a complete abTCR and interact with MHC/peptide expressed by DC and TEC. Cells with TCR affinity for MHC Class I down-regulate CD4 and acquire CTL potential, while MHC Class II-reactive cells lose expression of CD8 and become helper T-cells. Negative selection occurs when the T-cell interacts strongly with self-MHC/peptide on DC or Aire-expressing mTEC, and is induced to apoptose to prevent autoimmunity. Mature CD4+ or CD8+ T-cells exit the thymus at the CMJ.

The lymphoid compartment of the thymus does not contain long-term self-renewing haemopoietic stem cells (HSC) and therefore must be continually seeded by the bone marrow (BM). Heterogeneous BM progenitors have been found to circulate in the blood, including LSK Flt3$^+$ multipotential progenitors (MPP), ELP and circulating T-cell progenitors (Lin-Thy-1$^+$CD25$^+$; CTP) (Wright et al. 2001; Schwarz et al. 2004; Perry et al. 2006; Krueger et al. 2007), however, the precise phenotype of thymic immigrants and their level of extrathymic commitment to the T-cell lineage remains complex and controversial. It seems likely that a degree of redundancy exists, such that seeding of the thymus may occur via multiple alternative T-cell progenitors (see reviews by Bhandoola et al. 2007, Heinzel et al. 2007).

Despite their heterogeneity, extrathymic progenitors that have acquired the competence to migrate into the thymus do so in a specific and regulated manner. From the time of vascularisation (late in embryonic development), progenitors enter the thymus at the cortico-medullary junction and interactions with stromal cells enable Notch signalling, which is essential for T-cell commitment and loss of alternative lineage potential (Radtke et al. 1999).

Thymocytes have traditionally been described according to their levels of CD4 and CD8 expression with thymocytes negative for both markers (double negative; DN) differentiating into the CD4+CD8+ double positive (DP) stage before acquiring functional differences as CD4+ T-helper or CD8+ cytotoxic single positive (SP) lineages. CD44 and CD25 expression separates the DN subset into DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44+CD25+) and DN4 (CD44-CD25-) subtypes, through which progression is regulated by an interplay of Notch1 and interleukin 7 (IL7) receptor signalling. More recently the DN1 population has been found to be quite heterogenous when subdivided according to CD117 (c-kit) and CD24 expression (DN1a-e), and whilst all can develop into T-cells, they differ in their kinetics, proliferative ability and lineage potential (Porritt et al. 2004). The earliest described precursors resident in the thymus and committed to the T lineage reside in the perimedullary cortical zone and are termed early thymic progenitors (ETPs) or DN1a cells. These are also quite heterogenous in their expression of Flt3 (CD135) (Sambandam et al. 2005) and CCR9 expression (Heinzel et al. 2007).

ETPs differentiate into DN2 cells, which are mainly T-lineage specified, but retain a limited potential to develop into natural killer (NK) and dendritic cell (DC) lineages (Wu et al. 1996; Shen et al. 2003), and migrate out through the inner cortex to the outer cortex. Here αβ or γδ divergence occurs, as αβ-destined DN3 cells undergo TCRβ chain selection which combines with the pre-Tα-chain to allow rescue from programmed cell death (death by neglect) and leads to blockade of further TCRβ chain rearrangement (allelic exclusion), enhancement of TCRα chain rearrangement, intense proliferation and differentiation into DN4 cells, and subsequently DP thymocytes.

DP cells in the deep cortex rearrange one TCRα allele to replace the pre-Tα chain in the TCR complex. If this new αβTCR cannot interact with major histocompatibility complexes (MHC) on cortical epithelial cells (cTEC), the cell will apoptose. This process is termed MHC restriction, and imposes a low degree of self-reactivity across the T-cell repertoire, to ensure peripheral T-cells will recognise antigen presented in the context of self MHC. To ensure that the T-cells will be nonresponsive to selfpeptides, they are exposed to both ubiquitously expressed selfantigens presented by DC and a special set of tissue restricted antigens transcribed by medullary epithelium (mTEC). αβTCR specificity for MHC Class I or II expressed on TECs and strength or duration of signalling dictates the divergence of CD4+ T-helper or CD8+ T-cytolytic cell lineages. Further lineage-specific factors such as Runx3 and cKrox and other as yet unidentified factors are involved in establishing the gene expression associated with cytolytic CD8 or helper CD4 lineages (reviewed in Aliahmad and Kaye 2006). Mature CD4+ and CD8+ T-cells exit into the periphery as naïve T-cells.

1.2 Thymic Development of NKT and CD4+CD25+ Tregs

In addition to production of αβT-cells, the thymus also regulates development of lymphocytes responsible for the regulation of immune responses—most notably natural killer (NK)T-cells, which can produce pro or antiinflammatory cytokines, and the antiinflammatory Tregs, defined by coexpression of CD4, CD25 and the FoxP3 transcription factor.

NKT-cells develop in the thymus alongside conventional T-cells and share the earlier stages of thymic development, but they do not undergo the same manner of positive selection. Instead, the invariant NKT-cell TCR (Vα14-Jα18 in mice and Vα 24-Jα 18 in humans) binds to as-yet undefined glycolipid antigens presented by the MHC Class I-like molecule CD1d on surrounding double positive thymocytes instead of classical MHC molecules expressed on cortical epithelial cells (Bendelac 1995; Coles et al. 2000; Gapin et al. 2001; Speak et al. 2007; Porubsky et al. 2007). Newly selected NKT-cells continue their maturation and upregulation of NK1.1 following activation in the spleen and liver (Pellicci et al. 2002). A population of NKT-cells appear to remain as thymic residents, presumably influencing thymopoiesis through their broad range of cytokines.

Tregs follow the normal path of thymic development and are selected as part of the natural $CD4^+$ T-cell repertoire as cells with moderate affinity for self. Unlike conventional T-cells, where a high affinity TCR interaction with MHC/peptide leads to clonal deletion, Tregs with sufficient self-affinity are positively selected and continue development (Jordan et al. 2001; Kim et al. 2006). In humans, production of thymic stromal lymphopoietin (TSLP) by the epithelial whorls known as Hassall's corpuscles induces DC to instruct CD4+ SP-cells to become FoxP3+ Tregs through interactions involving MHC class II, costimulatory molecules and IL-2 (Watanabe et al. 2005).

1.3 Thymic Stromal Cells: Support and Selection

Until recently, thymic stromal cells (TSC) were considered a static, resident population, but have since been revealed as a heterogeneous population capable of undergoing extensive remodelling and regeneration (Gray et al. 2006). The thymic microenvironment is a three dimensional network of interconnecting stromal cells divided into regions through which thymocytes migrate and develop. These niche stromal cells include epithelium (TEC); mesodermal fibroblasts; endothelial cells and a proportion derived from other tissues including neural cells, myocytes and adipocytes (Boyd et al. 1991; van Ewijk et al. 1991; Gray et al. 2002; Anderson et al. 2001; Anderson et al. 2006). Just as the stroma directs thymocyte development, signals from the developing thymocytes also function to maintain these thymic stromal compartments in a dynamic codependence known as thymic cross-talk (van Ewijk et al. 2000; Anderson et al. 2001; Klug et al. 2002).

Demonstrating their important function as antigen presenting cells (APC) to developing thymocytes, all TEC express MHC Class II and can be further divided on the basis of MHCII expression levels (e.g., mTEC-high). The Autoimmune Regulator, Aire, is a transcription factor expressed by a subset of mTEC-high cells, which directs intrathymic expression of a set of peripheral antigens, resulting in a T-cell repertoire purged of clones reactive to organ-specific, late-onset and sequestered antigens (Anderson et al. 2002; reviewed by Anderson et al. 2006). An Aire deficiency results in autoimmunity in several models, associated with reduced expression of peripheral antigens. However, the role of Aire is likely to extend to chemokine expression. A recent study by Gillard et al. (2007) also showed reduced numbers and altered phenotypes of mTEC subsets in Aire-deficient mice, which may indicate an additional role for Aire, or may occur as a result of perturbed thymocyte migration and selection.

Although T-cell production throughout life is maintained by constant immigration of blood-borne progenitor cells, the maintenance of the epithelial microenvironment is not well understood. Fibroblasts are known to stimulate TEC differentiation and proliferation through provision of growth factors including fibroblast growth factor 10 (FGF10) and keratinocyte growth factor (KGF; also called FGF7) (Jenkinson 2003; Gray 2007). However, if the adult thymus contains epithelial stem cells as described in the embryonic (Gill et al. 2002; Bennett et al. 2002; Rossi et al. 2006) and early post-natal thymus (Bleul et al. 2006), as well as other adult tissues (Young et al. 2005), this is yet to be clearly demonstrated.

More recently we have shown that the progenitor capacity of TEC defined by expression of the MTS24 antigen is lost late in embryogenesis, and that high cell numbers of MTS24– cells (~100 fold that of MTS24+ cells) have the ability to form a thymus after in vitro aggregation (Rossi et al. 2007a). These data are consistent with the presence of multiple TEC progenitor cells, the MTS24+ cells being more efficient earlier in development. Accordingly, the swift regeneration of thymic epithelium in young adult mice after chemotherapy-induced involution certainly suggests the presence of one or many TEC populations readily able to replace damaged TEC subsets.

2 The Clinical Relevance of Thymic Atrophy

Long before the function of the thymus was known, it was observed at autopsy to be profoundly smaller in adults compared to children. Later studies confirm changes in cellular organization and composition with age, and describe in humans a strong inverse correlation between the rate of T-cell export and age (Mackall et al. 1995; Aspinall et al. 2000; Flores et al. 1999). Reasons for this loss of thymic mass and function are unknown, but this phenomenon is highly conserved amongst mammals, and it seems likely that evolutionary pressures shaped our physiology to reduce the energy cost of a T-cell production line at unnecessarily high level, or to reduce the likelihood of aberrant T-cell production once the

peripheral repertoire has been established. The atrophied thymus does maintain some activity; however, T-cell output is greatly reduced (Bertho et al. 1997). This incompletely understood phenomenon results in reduced numbers of naïve T-cells in the peripheral T-cell pool.

Immunosenescence is the state of impaired immune responsiveness that contributes to the increased susceptibility to infection, cancer and autoimmune diseases observed in the aged (Pawelec et al. 1997). Thymic involution precedes age-related impairment of peripheral T-cells, which contribute heavily to a progressive loss of cell-mediated immunity (Hirokawa et al. 1984; Ginaldi et al. 1999; Aspinall et al. 2000). Whilst thymic function is still evident in the aged, there is a large decrease in TCR-excision circle (TREC) levels; a measure of TCR gene rearrangement, and therefore of naïve T-cell output (Douek et al. 1998; Jamieson et al. 1999; Douek and Koup 2000).

In adults, the thymic contribution to the lymphocyte pool is dwarfed by the homeostatic, clonal expansion of preexisting memory T-cells (Berzins et al. 1998; Scollay et al. 1980; Haynes et al. 2000; Wack et al. 1998). These cells proliferate in response to infections throughout life and are retained at the expense of other mature polyclonal T-cells (Khan et al. 2002; Sansoni et al. 2007; Colonna-Romano et al. 2007). Remnant thymic function is important, even in the aged, since newly generated CD4 T-cells can function well in aged mice, while memory T-cells generally do not (Haynes et al. 2005); in aged patients, memory T-cells commonly show severe functional deficiencies (Haynes et al. 2003; Sansoni et al. 2007).

As a consequence of this homeostatic proliferation to maintain the T-cell pool in the aged, there is restriction of the peripheral TCR Vβ repertoire and CDR3 length distribution in old mice and elderly humans, indicating a reduction in the diversity of the T-cell pool (Schwab et al. 1997; Mosley et al. 1998; LeMaoult et al. 2000). Since the TCR has been randomly generated in each naïve T-cell, their overwhelming lack in the elderly translates to a reduced likelihood that the body will be able to respond to new pathogenic challenge.

T-cell anergy or exhaustion is a demonstrated outcome following prolonged activation or lymphopenia-induced T-cell proliferation, particularly relevant in chronic viral infections such as HIV, Hepatitis C and CMV (Day et al. 2006; Urbani et al. 2006; Sansoni et al. 2007). With age, there is evidence of decreased T-cell dependent antibody production, generation of allospecific cytolytic T-cells, and T-cell responses to mitogen or Ag stimulation (Hertogh-Huijbregts et al. 1990; Bloom et al. 1994; Nicoletti 1994).

Continued peripheral turnover of memory T-cells also increases the chance of incorporation of errors during division, which could plausibly result in cancer, or autoimmunity. Both are associated with defective immune function and lymphopenia, and both increase in frequency with age (DePinho 2000; Prelog 2006). The output from the atrophic thymus can potentially compound these risks, since the disrupted thymic microenvironment may no longer permit sufficient interaction between TSC and thymocytes, resulting in defective negative selection and a breakdown in T-cell tolerance.

2.1 Acquired Immunodeficiencies and Atypical T-cells: the Atrophic Thymus Fails to Meet Peripheral Demand

The most clinically significant insults to T-cells and the immune system in general are HIV/AIDS, causing death from overwhelming infection, and chemotherapy or radiation therapy, which form the standard of care for cancer patients. In the USA alone, there are over one million patients receiving chemotherapy annually and over 40,000 patients requiring either autologous or allogeneic HSC transplantation following high-dose cytoreductive myeloablation. These treatments would be applied still more broadly if not for the time taken to restore immunity in the adult, and the inherent risk of life-threatening infection.

Under adverse circumstances where peripheral T-cells are depleted, a functioning thymus is still absolutely required for the generation of new αβ T-cells (reviewed by Mackall and Gress, 1997; Haynes et al. 2000; Berzins et al. 2002). The rate of export of mature, naïve T-cells from the thymus is indexed to thymus size (Scollay et al. 1980) and studies by Berzins et al. (1998) demonstrated that the rate of thymocyte export is not regulated by the size of the peripheral T-cell pool. Thus, a reduction in peripheral T-cells does not trigger increased T-cell production, and the subsequent reconstitution from an aged, atrophied thymus will take many times longer than from a young thymus. Adults that show low levels of thymic export have markedly delayed peripheral T-cell regeneration after BMT, which is associated with a greater incidence of opportunistic infections compared to their younger counterparts whose thymus may be up to 10 times more effective (Scollay et al. 1980; Heitger et al. 2000). There is also a strong correlation between age and the recovery of phenotypically naïve T-cells and TREC levels following chemotherapy and BMT; and between age and viral load in HIV+ patients (Douek et al. 1998; Heitger et al. 2000; Douek et al. 2000; Weinberg et al. 2001). The robust link between the rate of T-cell recovery, age and survival in these patients (Mackall et al. 1995; Fassas et al. 2002) emphasises the relevance of thymic regeneration, and is the driving force behind several promising therapeutic approaches to increase the thymus size, its rate of function, and therefore T-cell export.

A study of parameters indicative of thymic function (SjTREC, CD45RA+ and Vβ repertoire profiles) after HSCT in patients over 30 years of age highlighted the importance of thymic-dependent regeneration in restoring CD4 T-cells (Hakim et al. 2005). In this study, the capacity to restore thymopoiesis was inversely proportional to age, and patients lacking evidence of thymic activity were still CD4 T-cell deficient up to 5 years posttransplant. Thus, although the atrophied thymus retains some minor function under normal conditions, with age, there are limits to the damage it can sustain, which makes it a valid and important candidate for regenerative therapy.

Postimmunodepletion, CD8 T-cells from aged mice and patients recover as an atypical population as a consequence of peripheral expansion. This homeostatic process generates CD8+ cells that are CD28- and CD57+, have restricted TCR repertoires, and are probably derived from a limited number of oligoclonal cells

(Posnett et al. 1994; Gorochov et al. 1994; Mackall et al. 1996). The lack of CD28 is important, as it denotes a state of functional anergy in these cells resulting in increased susceptibility to antigen-induced cell death if repeatedly stimulated (Posnett et al. 1999; Borthwick et al. 2000). The expansion of CD8+CD28- cells is seen in a number of clinical settings associated with impaired thymic function including: BMT or HSCT (Mackall et al. 1997; Muraro et al. 2005), GVHD (Fukuda et al. 1994), HIV infection (Brinchmann et al. 1994) and even simply as a consequence of ageing (Sansoni et al. 1993; Posnett et al. 1994; Fagnoni et al. 1996; Nociari et al. 1999; Brzezinska et al. 2004; Sansoni et al. 2007). Painstaking studies in aged patients have convincingly shown that the expansion of severely restricted oligoclonal memory T-cells probably occurs in response to chronic viral infection, and that the severity of age-related immune defects correlates with low life expectancy (Khan et al. 2002; Wayne et al. 1990).

Collectively, these changes have an enormously detrimental impact on the ability of the peripheral T-cell pool to combat pathogens. Thus, any mechanism by which thymic function could be improved would be of overwhelming clinical importance to the growing numbers of immunosuppressed patients, with the potential to effect a real difference in survival rates due to improved immune restoration.

3 Sex Steroid Ablation (SSA) as an Immunoregenerative Therapy

3.1 Sex Steroids Drive Thymic Atrophy

In both rodents and primates, the thymus is at its largest prior to adolescence. Although evidence suggests that atrophy is initiated earlier in humans (Steinmann et al. 1985; Bertho et al. 1997), increased circulation of sex steroids at puberty marks the beginning of a profound and steady degeneration (Hirokawa et al. 1994; Tosi et al. 1982; Windmill and Lee 1999), resulting in reduced lymphoid and stromal tissue, with progressive loss of structural integrity and organisation. The thymus also undergoes a transient involution in response to stress or pregnancy, induced by an increase in glucocorticoids or sex hormones respectively. Thymic recovery after stress and postpartum is rapid, unlike age-related involution, which is chronic (Luz et al. 1969; Nabarra and Andrianarison, 1996).

Animal castration studies demonstrate a profound hypertrophy of the aged thymus relative to bodyweight (see Fig. 2), which is reversible by testosterone injection; results confirming the causal link between onset of puberty and thymic atrophy (Grossman 1984; Greenstein et al. 1986; Utsuyama et al. 1989). Later studies showed that the thymus is also enlarged in mice with defects in androgen action (Olsen and Kovacs,1989), so-calledandrogen-resistant testicular feminization (TFM)mice. These observations suggested that androgen exposure inhibits thymopoiesis, but the effect of hormone production on the immune system is anything but simple, governed by both positive

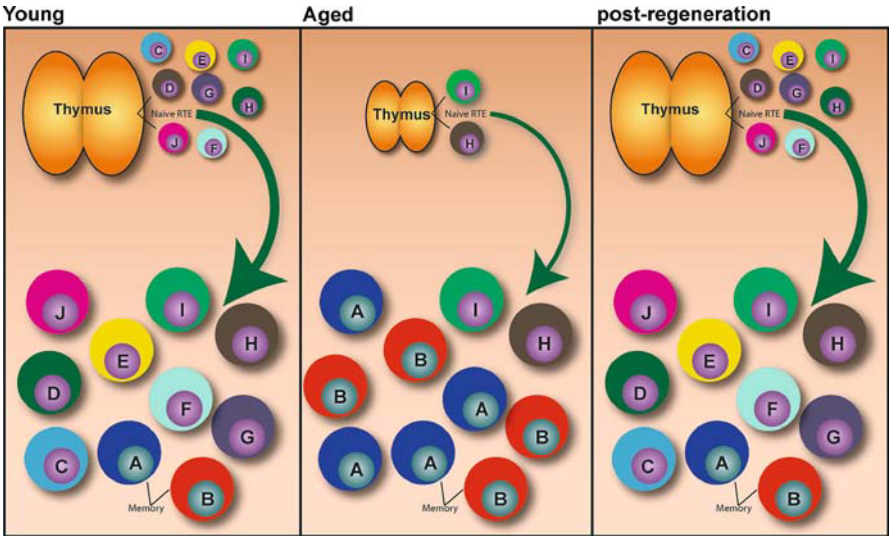

Fig. 2 Thymic regeneration reverses age-related narrowing of the T-cell repertoire. In young mice and humans, naïve recent thymic emigrants (RTEs) with a broad range of specificities for antigen (designated A-J) exit the thymus and are incorporated into the peripheral T-cell pool. In the aged, slow clonal expansion of memory T-cells with limited diversity (A, B) occurs as a likely response to chronic virus exposure. Since thymic output is limited, the T-cell repertoire narrows considerably. Following thymic regeneration, however, the output of new, naïve T-cells restores the breadth of the repertoire without affecting the capacity of remaining memory T-cells to respond upon antigen encounter

and negative feedback loops operating between the hypothalamus, pituitary gland, gonads, adrenal glands, thymus and bone-marrow (Fig. 3).

Sex steroid production begins with the hypothalamus, which releases luteinizing hormone-releasing hormone (LHRH, also referred to as GnRH), which acts on the LHRH-receptors (LHRH-R) in the anterior pituitary gland (Fig. 3a). The pituitary subsequently produces luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act on the gonads to produce testosterone or estrogen (Moghissi, 1990). Sex hormones then feed back to directly halt LHRH, LH and FSH secretion. Sex steroids dampen thymic activity, thymopoiesis and B lymphopoiesis, while LHRH imposes a direct stimulatory effect on lymphocytes (Grasso et al. 1998; Tanriverdi et al. 2005). Thus, the effect of surgical castration is to both remove the negative feedback from the sex steroids, while increasing the effect of LHRH due to the lack of testosterone feedback from the gonads (Belvisi et al. 1993).

Surgical castration reduces serum testosterone to approximately 1% of normal levels within six hours in male rodents (Kyprianou et al. 1988) and this allows for a more defined analysis of the early effects of SSA, compared to chemical castration by LHRH receptor agonists. A significant increase in thymic cellularity was evident

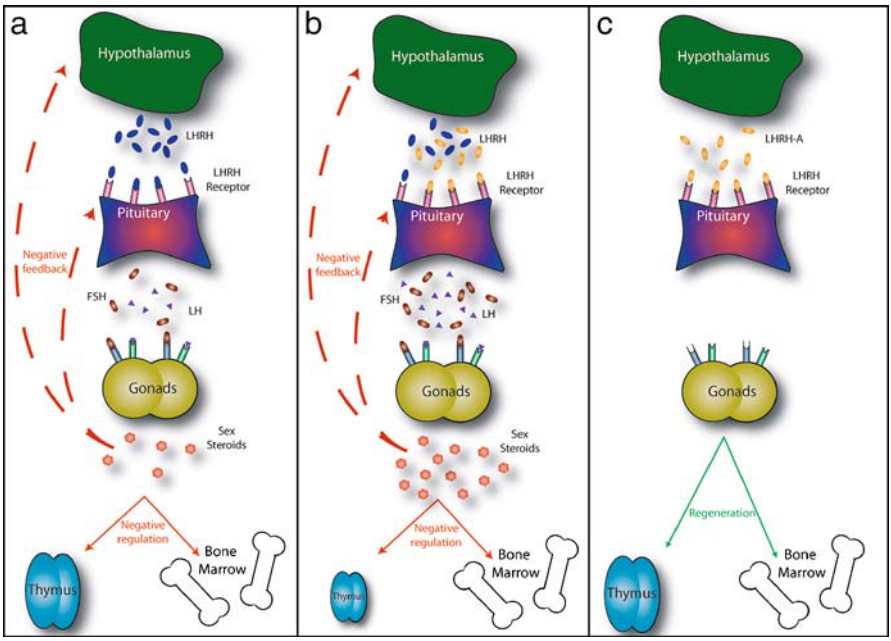

Fig. 3 The neuro-immune-endocrine axis and LHRH-agonist treatment. Throughout reproductive life, LHRH is produced in pulses from the hypothalamus, binding to the LHRH-receptor on the pituitary gland (panel a). The pituitary releases FSH and LH, which signal to the gonads to produce sex steroids, which are involved in negative feedback loops to the hypothalamus and pituitary, thus regulating the levels of hormones produced. Sex steroids also chronically inhibit lymphopoiesis in the bone marrow and thymus. Following administration of an LHRH-agonist (LHRH-A) (panel b), the LHRH receptor on the pituitary is continuously stimulated, leading to an initial surge in FSH and LH, and subsequent increase in sex steroid production. This effect is short-lived (panel c), as the continuous administration causes desensitisation of the LHRH-receptors and a cessation of sex steroid production. A lack of sex steroids results in regeneration of the thymus and bone marrow lymphopoietic activity to pre-pubertal levels

within three-five days of androgen blockade by surgical castration and a return to young levels was reached by around seven days with expansion continuing beyond young levels by ten days (Sutherland et al. 2005; Heng et al. 2005). In terms of the kinetics of regeneration, a proportional increase in the TN compartment was initially evident by day three-five followed by a proportional increase in DPs, significant by day seven. All TN subsets showed an increase in cell number at day five and importantly, the age-induced reduction in proportion and absolute cell number of ETPs was rapidly restored following castration, consistent with the evident increase in proliferation and reduction in apoptosis of these cells. CD4+ and CD8+ mature SP cells also showed an increase in proliferation although the absolute number of these cells was relatively constant, due possibly to migration of these cells into the periphery.

3.2 SSA Rejuvenates Thymic Stromal Cells (TSC)

Whilst many studies assess the effects of sex-steroid ablation upon thymocyte recovery and consequent peripheral T-cell recovery, few have focused on the effects upon the thymic stroma.

SSA restores both the morphological and numerical TSC defects seen in aged C57B6 mice. A significant reorganization of the TSC microenvironment can be seen at 2 weeks post castration, rendering aged stroma indistinguishable from the young adult thymus (Sutherland et al. 2005). Castration of 10 month-old mice results in marked stromal cell expansion, with significantly higher numbers of TECs after 7 days compared to sham-castrated controls (Gray et al. 2006). TEC exhibited significant increases in proliferation seven days post castration compared to sham-castrated mice, resulting in substantial cell increases in both the medullary and cortical epithelial compartment. Castration also reversed age-related changes in proportions of TEC subsets, resulting in expansion of the medulla and leading to the restoration of mTEC/cTEC and mTEC-high/mTEC-low ratios to normal young levels (Gray et al. 2006). TEC regeneration was reliant on cross-talk with thymocytes, as castration of RAG-/- mice in the same study showed that the removal of sex steroids is insufficient to drive TEC proliferation in the absence of the appropriate thymocyte subsets. There is some indication that early changes in the production of growth factors such as IL-7 and Growth Hormone by stromal cells postcastration contribute to initial thymocyte expansion (Ann Chidgey, unpublished observations). Interestingly, although the thymocyte numbers from aged mice reach normal young levels seven days after castration, TEC numbers increased 2-fold, but did not return to young levels (Gray et al. 2006).

Adult TEC subsets may be maintained by low level persistent self-renewal in normal homeostasis, but when destroyed by drugs or irradiation, it is possible that an additional progenitor compartment contributes to the epithelial restoration, driven in part by signals from mesenchymal cells. It is very likely that sex steroids initiate thymic atrophy through suppression of these TEC subsets. Whilst the beneficial effects of SSA can be seen in mouse TSC, effects upon the human TSC microenvironment have yet to be ascertained.

3.3 SSA Speeds Thymic Recovery After Chemotherapy, Beginning with the Earliest Thymocyte Subsets

Cyclophosphamide (Cy) is an alkylating agent used clinically to kill rapidly dividing cells in the treatment of cancer and as a preconditioning regime prior to BMT. Cy treatment of young mice kills the majority of leukocytes in blood and secondary lymphoid organs, causing immunosuppression. The thymus is greatly damaged, and in young mice, thymic cellularity drops 50-fold within 3 days of treatment (Heng

et al. 2005). Restoration of peripheral T-cells must therefore begin with thymic regeneration; SSA provides a dual stimulus to immune recovery postchemotherapy by inducing faster thymic growth followed by sustained, increased T-cell output (Heng et al. 2005).

DP and TN thymocytes are most strongly affected by Cy treatment. We have shown that at the time of greatest involution, 3 days post-Cy treatment, mice castrated one day prior to injection have significantly higher numbers of TN-cells, and an overall increased thymus size, which reaches almost double the size of sham castrated control mice by 5 days post-treatment but is significantly higher at all timepoints assessed in the study (Heng et al. 2005).

Further dissection of the TN subset, which contributed solely to the increased thymic cellularity in castrated mice until 5 days post-Cy treatment, showed that the numbers of TN2, TN3 and TN4 cells were all increased at day three, and that a wave of development in the castrated mice drove increased TN2 cells at day 3, creating increased TN3 cells at their highest on days 4 and 5, and TN4 cells peaking at days 5 and 6 compared to sham-castrated mice. It was not possible to tell from this study whether the TN1 subset had been increased prior to day three leading to the increase in TN2 cells; whether the increases in TN2-4 subsets were due to proliferation alone, independent of TN1 seeding; or whether cells in castrated animals progressed faster through TN1 to TN2-4 in this model of thymic damage. The TN1 subset, like all TN cells, showed increased proliferation in the castrated group (Heng et al. 2005).

Hence, thymi from castrated mice recovered significantly faster than sham-castrated mice, the overall pattern of recovery mimicking that of normal thymocyte development, with a wave of increased TN cells (highest at day 4 in both castrated and sham-castrated mice) giving rise to increased DP cells (days 6 and 7), followed by increased SP thymocytes at 2 weeks post-treatment (Heng et al. 2005).

3.4 Enhanced Thymic Epithelial Cell Recovery after SSA Therapy

Treatment of young mice with cyclophosphamide not only decreases thymocyte numbers but causes profound loss of thymic stroma. Microscopy studies show that, in young rats, the reticulo-epithelial network recovers four weeks after cyclophosphamide-induced acute thymic involution (Yoon et al. 2003), with noticeable alterations in RER and Golgi apparatus consistent with high synthetic activity (Yoon et al. 1997).

Although young animals have the inherent ability to recover thymus function, castration is able to enhance this further. When young mice were castrated concomitantly with cyclophosphamide treatment, resulting in acute involution of the thymus, TEC were equally affected in both castrated and sham-castrated mice, but in all cases, TEC recovery was faster in castrated mice (Daniel Gray, Natalie Seach, unpublished data).

Significantly, cyclophosphamide treatment causes a profound loss of mTEChi cells and Aire expression (Anne Fletcher, Natalie Seach unpublished data). Given the recent studies by Rossi et al. (2007c) demonstrating the importance of RANK signalling in maintenance of Aire expression, the upregulation of RANKL expression by subcapsular, paratrabecular, perivascular and medullary TEC which occurs during thymic regeneration after cyclophosphamide treatment (Lee et al. 2005) conceivably serves to restore Aire expression, as well as supporting increased differentiation of early thymocytes. In castrated animals, mTEChi cells recover significantly faster compared to sham-castrated controls, suggesting normal negative selection is restored faster, in parallel with faster recovery of medullary thymocyte populations (Natalie Seach, unpublished data).

4 Clinical Removal of Sex Steroids for Thymic Regeneration

4.1 Reversibly Reducing Sex Steroids

To be clinically indicated as an immune recovery therapy, studies performed to date suggest that the relevant agent must be capable of reducing sex steroids to castrate levels; whether subcastrate levels can be effective remains to be tested. The treatment must also be fully reversible. Drugs currently licensed for clinical use to reduce sex steroids include those blocking normal LHRH activity (LHRH agonist or antagonist), and sex steroid blocking agents including flutamide and bicalutamide (which competitively bind to androgen receptors) or tamoxifen (a selective estrogen receptor modulator).

Chemical castration involves either reversible desensitisation or blockade of the LHRH-R by long-term administration of an LHRH agonist (LHRH-A) or antagonist respectively. LHRH is usually produced in pulses from the hypothalamus, due to feedback from the gonadal sex steroids. Constant administration of an LHRH-A overrides the cyclic signalling, causing an initial surge of sex steroid production (Fig. 3b) before effectively down-regulating the LHRH-R on the anterior pituitary, and subsequently blocking secretion of LH and FSH (see Fig. 3c). Circulating sex steroids fall to castrate levels in approximately 3 weeks using the LHRH-A (Haisenleder et al. 1987; Filicori and Flamigni, 1988). The antagonist simply blocks the LHRH binding site and prevents signalling. LHRH-A are commonly used to treat a variety of conditions, including sex steroid-sensitive malignancies such as breast and prostate cancers; precocious puberty and endometriosis (Huben, 1992; Neely et al. 1992; Waller et al. 1993). Both LHRH agonists and antagonists dramatically rejuvenate the thymus in rodents and also in humans (Greenstein et al. 1986; Windmill et al. 1993; Sutherland et al. 2005).

Low levels of sex steroids, particularly estrogen in females, are also produced by the adrenal glands; this process increases with age and is unaffected by LHRH-A administration. Ovariectomised rats show a gradual increase in levels of androgens produced by the adrenal cortex. These are converted to estrogens by aromatase

at extragonadal sites including adipose tissue, bone, muscle and brain, resulting in an increase in serum estradiol, compared to intact rates, from 7% immediately postsurgery, to 50% (Zhao et al. 2005). Although LHRH-A administration does not affect this process, androgen or estrogen receptor blockade can overcome any effects of these ectopically produced steroids, and could be coupled to the use of LHRH-A. The use of androgen or estrogen blockers alone would not have the added direct stimulatory benefit to the immune system that LHRH-A seems likely to mediate.

An important clinical consideration is the requirement for remnant thymic function as a basis for regeneration. Studies in mice have shown that although castration increases thymus size and output in mice as old as 2 years, with effects persisting for at least 12 months (Sutherland et al. 2005), the thymus of castrated 24 month old mice is still smaller than castrated nine or 18 month old mice (Reiseger et al. manuscript in preparation). This finding demonstrates the complex mechanisms governing thymic atrophy and regeneration, suggesting that sex steroids are not solely responsible for age-induced thymic involution. Quantitative and qualitative changes in lymphoid progenitors are also likely to be involved.

4.2 Sex Steroid Receptors and Mechanisms of Thymic Regeneration

The thymus expresses many hypothalamic and pituitary hormones and hormone receptors (Table 1). The neuro-thymic-endocrine axis is complex and poorly understood, with the expression of a hormone or its receptor potentially able to mediate either inhibitory or stimulatory processes. Administration of an estrogen receptor blocker in the neonate results in reduced thymic development (Staples et al. 1999), although this effect could clearly be indirect with many growth patterns affected by estrogen. In addition, sex steroid receptors in immune cells differ in their intracellular location (cell-surface; nuclear), expression pattern and signalling pathways, making prediction of their precise intrathymic function difficult. However, studies of hormone and receptor expression patterns can certainly identify cell types most likely to be affected by SSA and LHRH-A administration, and PCR studies of sex steroid and LHRH-receptors on specific stromal cell subsets can help to further identify early effector populations during thymic regeneration.

The target cells of sex steroids have been identified through expression patterns of androgen receptors (AR). Early studies found AR expression on thymocytes and TEC (Olsen et al. 2001) with the latter showing 6-fold higher expression (Kumar et al. 1995). Unpublished data from our laboratory shows that AR are expressed by all subsets of thymic stromal cells, including DCs and CD31+ endothelial cells, and that although TEC expressed high levels of AR, mesenchymal fibroblasts showed 2-fold higher expression (Tomoo Ueno, unpublished observations). The broad expression pattern of AR immediately suggests that the mechanisms of thymic regeneration are unlikely to be confined to one driving cell type nor a simple mechanism. We are

Table 1 Intrathymic expression of hypothalamic, pituitary and gonadal hormones and receptors

	Thymocytes	Thymic stroma
LHRH[a]	+	TEC
LHRH-R[a]	+	?
LH[a]	+	?
GH[a, b]	+	TEC
GH-R[a, b]	DN >> DP > CD4, CD8 SP	TEC
AR[a]	+	Fibroblasts > TEC > endothelium, DC
ER[c, d]	+ lower levels than TEC	Subcapsular TEC and corticomedullary junction mTEC
PR[d]		Subcapsular TEC and corticomedullary junction mTEC
ACTH[e]	+	TEC
PRL[e]	+	-
PRL-R[e, f]	DN >> CD8+SP > DP, CD4+SP	TEC
OT[e, g]	-	Subcapsular TEC, cTEC
OT-R[e, g]	DN, DP, CD4+SP, CD8+SP	TEC
VP[e, g]	-	Subcapsular TEC, cTEC
VP-R[e, g]	Only V3R is expressed, and only in DP and CD8+SP	TEC

LHRH: luteinizing hormone releasing hormone (GnRH). LHRH-R: LHRH receptor. LH: luteinizing hormone. GH(-R): growth hormone (receptor). AR: androgen receptor. ER: estrogen receptor. ACTH: corticotropin. PRL (-R): prolactin (receptor). OT (-R): preprooxytocin (receptor). VP(-R): preprovasopressin (receptor).

[a] determined by q-PCR; unpublished data, Boyd laboratory

[b] de Mello-Coelho et al. 1998 Kawashima et al. 1995

[d] reviewed by Li et al. 2002

[e] reviewed by Savino et al. 1999

[f] Gagnerault et al. 1993

[g] reviewed by Hansenne (2005)

+ expressed; - not expressed; ? unknown

thus currently performing gene chip analysis on purified stromal cell subsets to identify some of the main pathways for thymic regeneration.

Chimeric experiments using testicular feminization (Tfm/y) mice demonstrated that androgen receptor signalling in nonhaemopoietic stromal cells most affected thymic involution (Olsen et al. 2001).Tfm/y mice express a defective AR in both lymphoid and nonlymphoid thymic compartments and show significant thymic enlargement, which was not decreased by androgen administration. Chimeric studies showed that mice with AR+ haemopoietic compartment but AR- stromal cells were also resistant to thymic atrophy, suggesting a role for TSC in initiation of this process. Conversely, thymi expressing the AR on stromal cells but not thymocytes involuted following testosterone administration. However, castration only modestly increased

thymic size in these AR deficient mice, suggesting that although atrophy is initiated by the stromal cells, regeneration also requires some direct effect on thymocytes or BM precursors, most likely a positive feedback loop which requires expansion of progenitors and downstream thymocytes in order to expand stromal cells.

Mechanisms of thymic regeneration in female mice have not been extensively studied, however, 2 distinct forms of the estrogen receptor (ER), ERa and ERb, seem likely to play opposing roles. Experiments using selective agonists for each receptor indicate that signalling through the ERa not the ERb causes thymic atrophy. ERa agonist administration also altered the CD4/8 profile in the thymus, while the ERb agonist, when administered together with ERa agonist appeared to partially offset the atrophic effect of ERa signalling (Li et al. 2006).

Growth hormone (GH) and IGF-I are strong candidates for mediating thymic regeneration post-SSA, as they decrease in production with age and injection of recombinant human GH regenerates the thymus and results in increased IGF-I secretion (Taub and Longo 2005), possibly through stimulation of the TEC-produced growth factor thymulin. Other feasible candidates include proproliferative and antiapoptotic molecules such as IL-7 and keratinocyte growth factor (KGF), which have both been shown to induce thymic regrowth in mice (Min et al. 2002; Pido-Lopez et al. 2002), or reductions in proapoptotic molecules such as TGF-β. However, in thymic regeneration post-castration and BMT, a preliminary study using whole thymic stromal preparations found no alterations in IL-7, TGF- , or KGF mRNA (Sutherland et al. 2005). Furthermore, administration of IL-7 does not reverse age-related thymic atrophy (Sempowski et al. 2002; Pido-Lopez et al. 2002) and thymic regeneration occurs after castration of KGF-/- mice (Gabrielle Goldberg, unpublished observations).

Nerve growth factor (NGF) induces angiogenesis via expression of VEGF in TEC, but does not appear to be causative in thymic involution. Levels of nerve growth factor NGF receptors p75LNGR and TrkA were reduced with age in TEC, but administration of NGF did not prevent thymic involution (Garcia-Suarez et al. 2000).

Based on current evidence, it is only possible to speculate on the molecular mechanisms of thymic regrowth after SSA, and further more detailed analyses are clearly required.

5 The Effect of SSA on Recovery of Peripheral Immune Function in Mice and Humans

5.1 Reversal of Age-related Changes in T-cell Proportions

The peripheral CD4+/CD8+ T-cell ratio undergoes profound alterations during chronic immune responses: allograft rejection; graft-versus-host disease; hemophilia (Menitove et al. 1983; Zander et al. 1985); and chronic infections such as cytomega-

lovirus, Epstein-Barr virus, and influenza virus (Carney et al. 1981; Crawford et al. 1981; Maher et al. 1985). There are varied reports on changes in CD4+ and CD8+ T-cell numbers with age that depend on species, strain, age and organs examined. In mice there are reported decreases in the CD4/CD8 T-cell ratio (Boersma et al. 1985; Grossmann et al. 1990; Callahan et al. 1993; Toichi et al. 1997; Berzins et al. 1999) and other studies which show no change (Sidman et al. 1987; Komuro et al. 1990; Kischmann and Murasko 1992), whilst in humans, both increases (Utsuyama et al. 1992; Schwab et al. 1997) and decreases (Pawelec et al. 1999) in the CD4/CD8 ratio have been reported with age.

Studies from our laboratory reported a reduction in the proportion of CD4+ T-cells with age, and found that SSA normalises the CD4:CD8 T-cell ratio in mice and humans by increasing the proportion of naïve T-cells at the expense of memory T-cells (Sutherland et al. 2005). Immune changes are not entirely thymus-dependent, however. Numerous studies in mice and humans have shown that androgens signal directly through AR on peripheral T-cells, decreasing proliferation and increasing apoptosis (Samy et al. 2000; Benten et al. 2002; Araneo et al. 1991; McMurray et al. 2001). In mice, SSA—whether via surgical castration or LHRH-A administration—has been shown to expand secondary lymphoid organs and improve T-cell function, including proliferation in response to mitogen (Windmill and Lee 1999), specific antigen and TCR/costimulation (Roden et al. 2004) and the CTL response to influenza (Reiseger et al. manuscript in preparation). Although the changes, if any, in the CD4/8 ratio with age remain contentious it is clear that the numbers of naïve T-cells in the periphery do decrease. SSA is able to reverse these changes and increase naïve T-cells. This is evident in prostate cancer patients, who following treatment with LHRH-A or AR blockade show increased naïve CD4+TREC+ levels (Sutherland 2005). SSA also results in enhanced proliferation in response to mitogen in endometriosis patients (Hsu et al. 1997) and reduced immunosuppression after haemorrhage or burn injury (Messingham et al. 2001).

Increased peripheral lymphocyte numbers are largely responsible for improved immune function following LHRH-A treatment (Garzetti et al. 1996; Oliver et al. 1995; Umesaki et al. 1999). Sutherland et al. (2005) showed that this increase was predominantly due to increases in T-cells linked to increased thymic output, but may, in part, be due to direct stimulation of peripheral T-cells by LHRH-A (Jacobson et al. 2004), This may be a temporary effect of the initial surge in sex steroids, which occurs soon after treatment with the LHRH-A, since expression of LHRH-R mRNA in peripheral lymphoid organs mirrors both LHRH and LHRH-R mRNA expression in the hypothalamus and pituitary (Jacobson et al. 1998), and therefore it is likely that these receptors on lymphocytes are down-regulated and desensitised after prolonged exposure to LHRH-A.

Castro (1974) showed that castration increased immune reactivity to sheep red blood cells and enhanced skin graft rejection, which was reversed by androgen administration. When mice were thymectomised and castrated, however, the skin grafts were accepted, indicating that the effects of sex steroids are linked to thymic output. Castro (1974) also showed delayed development and decreased incidence of methylcholanthrene-induced tumour following castration, which was abrogated

by thymectomy. Together with research demonstrating that speed of regeneration of peripheral T-cells following immunodepletion in humans is directly related to thymic size and output (Douek et al. 2000; Hakim et al. 2005), these findings have lead to the postulation that the observed effects on peripheral T-cell immunity after castration are predominantly due to alterations in the thymus, especially the increase in naïve T-cell output.

5.2 LHRH has Direct Stimulatory Effects on T-cells

Through removal of negative feedback on the hypothalamus and pituitary, and due to the broad expression pattern of neuroendocrine receptors on immune cells, the removal of sex steroids has multiple effects. Androgens suppress LHRH production and responsiveness within the hypothalamus and pituitary (Jennes et al. 1995) and androgen deprivation (AD) increases circulating levels of prolactin, LH and estradiol (Verhelst et al. 1994). LHRH directly increases T-cell proliferation and cytokine production in both men and women (Grasso et al. 1998; Tanriverdi et al. 2005). Thus, the ability of SSA to enhance T-cell levels and responses may be mediated in part by changes in the levels of other hormones such as LHRH and prolactin, rather than simply due to the lack of stimulation of sex steroid receptors (Marchetti et al. 1989; Jacobson et al. 2004; Buckley, 2001).

Production of LHRH in the thymus and spleen mirrors hypothalamic LHRH and is regulated by the same sex-steroid feedback mechanisms (Jacobson et al. 1998). Studies in rats showed that administration of an LHRH-A to middle-aged animals decreased the binding capacity of LHRH-R for LHRH-A in the thymus by 50%, including a 65% reduction in the number of receptors, while in aged rats, thymic binding of LHRH-A was completely abrogated (Marchetti et al. 1989), suggesting that any direct effect of LHRH-A administration on the thymus is likely to be short-lived. These authors also found that castration increased LHRH-A binding in the thymus, suggesting increased receptor expression. Jacobson et al. (1999) showed that administration of exogenous LHRH (rather than an agonist) did not change LHRH-R expression in immune organs of castrated male autoimmune-prone mice, but did affect their responsiveness to this hormone through increased G-protein signal transduction.

5.3 SSA Rejuvenates Bone Marrow and B Lymphocytes

Although not a focus of this review, in addition to its clinically promising effects on the thymus and T-cell immunity, SSA results in striking alterations to the bone marrow and B-cell development.

Similarly to T-cells, the production of B-cells is regulated in part by physiological levels of androgens and estrogens. Sex hormones suppress B-lymphopoiesis and augmentation occurs when their levels are decreased (reviewed by Kincade et al. 2000).

As such, SSA augments B-cell production and function, through direct effects on B-cells themselves as well as increasing the number of LSK-cells post-BMT (Sutherland et al. 2005) and early B-cell precursors (Goldberg et al. manuscript in preparation).

AR have been reported on BM stromal cell lines (Sakagami et al. 1993) and pro-B-cell lines and thus the effects of androgens on B-lymphopoiesis may be mediated directly on the B-cells or indirectly via the stroma (Viselli et al. 1997). Treatment with dihydrotestosterone (DHT) leads to a reduction in the numbers of IL7 responsive B-cell precursors (Smithson et al. 1998). Removal of androgen on the other hand results in significant increases in B-lymphopoiesis, as shown in castrated mice, mice with mutations in the AR (Tfm) and in mice with deficiencies in sex steroid production (Smithson et al. 1994, Viselli et al. 1997). Castration also improves B-cell function, by significantly increasing the specific antibody titre to Hepatitis B virus (Jessica Reiseger, manuscript in preparation).

Although the mechanisms by which sex steroid ablation improves B-lymphopoiesis are still unclear, the consequences for peripheral B-cells are significant and, as with T-cells, likely to speed recovery from chemotherapy in a clinical setting.

5.4 SSA, Tolerance and Autoimmunity

While restoration of T-cell function is clearly desirable, there will be a major propensity towards autoimmunity if normal tolerance mechanisms are not also reinstated. The high incidence of autoimmunity in Western society (over 3%, and rising) makes it a very real consideration when manipulating the immune system in any way (Jacobson et al. 1997). However, despite over 25 years' clinical experience with LHRH-A, there has been no link to increased levels of autoimmunity, suggesting that normal tolerance mechanisms are restored. Accordingly, in the thymus, the increases in thymocytes after castration are accompanied by proportional increases in DCs and mTECs (Sutherland et al. 2005; Gray et al. 2007) responsible for negative selection of autoreactive cells and positive selection of regulatory T-cells. In addition, wildtype mouse strains do not develop autoimmunity or GVHD following castration and HSCT, which is one indication that the immune system can regenerate normally after androgen depletion (Goldberg et al. 2007). However, numerous studies have shown increased peripheral T-cell function after SSA (Goldberg et al. 2005; Viselli et al. 1995; Viselli et al. 1997; Ellis et al. 2001; Olsen and Kovacs 2001; Wilson et al. 1995; Castro, 1974; Roden et al. 2004; Sutherland et al. 2005), which makes it important to ensure regulatory cells increase proportionally with effector T-cells.

The increase in the frequency of immune disorders and autoimmune diseases with age has been linked closely with quantitative and/or qualitative defects of cells within the regulatory arm of the immune response. Key players of immune regulation include $CD4^+CD25^+FoxP3^+$ Tregs, IL-10-producing $CD4^+$ Treg cells-1 (Tr1), TGF-β-secreting T-helper-3 cells (Th3), $CD4^+CD45RB^{low}$ T-cells, $CD8^+CD25^+$ Treg cells, γδ T-cells and natural killer T-cells (NKT) (Powrie et al. 1994; Thornton 2005; Hoglund 2006). The best studied of these are the thymus-derived $CD4^+CD25^+$ Tregs and NKT-cells.

5.4.1 CD4+CD25+FoxP3+ Regulatory T-cells (Tregs)

Naturally occurring Tregs exert active control over a variety of physiological and pathological immune responses (Sakaguchi 2004) and studies have shown that neonatal thymectomy at day 3 leads to a substantial reduction of Tregs in the periphery resulting in autoimmunity (Asano et al. 1996; Itoh et al. 1999).

Although thymic involution is conceivably expected to affect Treg generation similarly to other CD4 T cells, there is very little, variable evidence on the effects of immunosenescence on Tregs, and many studies have not employed FoxP3 staining to definitively identify this population. It is also unknown whether Tregs express AR, although indirect evidence suggests that they possess ER (Aluvihare et al. 2004; Polanczyk et al. 2004; Polanczyk et al. 2005; Arruvito et al. 2007) and a more recent study detected ERα in resting human Treg lysates (Prieto & Rosenstein 2006). Despite these limitations, in several studies, Treg prevalence and age appear to correlate (Brusko et al. 2005; Gottenberg et al. 2005; Gregg et al. 2005). Tregs are driven to expand in response to IL-2 (Almeida et al. 2006) and also proliferate in response to self-antigen (Cozzo et al. 2003; Walker et al. 2003) which makes it likely that although increased in number, the aged Treg TCR repertoire could be severely restricted due to homeostatic proliferation of pre-existing Treg cells.

Importantly, using intrathymic FITC injection, we have shown that increased peripheral Treg numbers post-SSA are due to increased thymic output in both male and female mice, showing that SSA acts on this cell type indirectly through thymic regeneration (Katerina Vlahos, manuscript in preparation). These findings are in contrast to a study in healthy male volunteers, which found that treatment with an LHRH antagonist decreased the percentage of CD4+CD25+ and CD4+CD25^{bright} T cells. However, this study did not report any effects on cell numbers and did not assess FoxP3 expression (Page et al. 2006).

5.4.2 Natural Killer T-cells

NKT cells are potent regulators of the immune system (Benlagha & Bendelac 2000) that influence diverse immune responses including the onset of autoimmune diseases such as type 1 diabetes, multiple sclerosis, SLE and rheumatoid arthritis (Hong et al. 2001; Jahng et al. 2001; Hammond et al. 1998; Lehuen et al. 1998) and the control of tumour growth (Cui et al. 1997; Smyth et al. 2000; Ambrosino et al. 2007). Functionally distinct subsets exist in humans and mice, and most CD4+ and DN NKT cells are thymic-dependent, while CD8+ NKT cells are only found in humans and develop extrathymically (Hammond et al. 1999; Kameyama et al. 2001; Pellicci et al. 2002). Resident populations in different organs can share a surface phenotype yet differ in function (Crowe et al. 2005).

Preliminary SSA studies on wild type (C57Bl/6) and autoimmune prone (NODLt and NZB) mice show that ovariectomy of wild type or NOD mice results in either no change or a decrease in peripheral thymic derived NKT cells respectively (Anne Fletcher, Samy Sakkal, Katerina Vlahos, unpublished observations) while castra-

tion results in either an increase or no change in NKT cells in wild type and NZB mice respectively (Katerina Vlahos, Anne Fletcher, manuscript in preparation).

The role of these cells in promoting tolerance, particularly in studies involving autoimmune models, is contentious and appears to vary depending on the genetic background and the inflammatory phenotype of the mice. Transfer or activation of NKT cells reportedly mediates protection in the NOD and EAE models of diabetes and multiple sclerosis respectively (Hammond et al. 1998, Leheun et al. 1998; Singh et al. 2001; Forestier et al. 2007) but endogenous NKT cells exacerbate disease in models of lupus (Forestier et al. 2005). In addition, there was no difference in the number or IL-4 producing capacity of blood NKT cells from type I diabetes patients (Lee et al. 2002). The significance of these findings with regard to the likely impact on tolerance restoration or continued anti-cancer immunosurveillance in humans recovering from immunosuppression is unclear. Certainly, evidence from both mice and humans exists to suggest that caution must be exercised when using the NKT cell levels in the blood as sole representative data, since limited correlation exists between blood and major organs (Berzins et al. 2004; Berzins et al. 2005)

5.4.3 Sex Steroids and Autoimmunity

The lower incidence of autoimmune disease in males compared to females is suggested to be due, in part, to host androgens (Grossman 1985; Whitacre et al. 1999), a theory supported by studies showing that androgens suppress the immune responses of vertebrates (for reviews, see Schuurs and Verheul 1990; Paavonen 1994; Olsen and Kovacs 1996). Conversely, exposure increases the susceptibility toward numerous infectious diseases (reviewed by Roberts et al. 1996). Exogenous androgen administration reverses the female-biased predisposition of NOD or NZB/W mice to developing diabetes and SLE-like autoimmune diseases respectively (Fox 1992; Roubinian et al. 1979; Roubinian et al. 1978; Fitzpatrick et al. 1991) and can abrogate immune responses against pathogen, allograft, or traumatised host tissue (Wichmann et al. 1996; Graff et al. 1969; Angele et al. 2000).

In accordance with these findings, androgen withdrawal in male mice can exacerbate the severity of various autoimmune disorders including EAE, SLE and insulitis and has been shown to potentiate a variety of host immune responses in various animal models (Fox 1992; Fitzpatrick et al. 1991; Bebo et al. 1999; Angele et al. 2000; Samy et al. 2000; Samy et al. 2001; Bellido et al. 1995; Keller et al. 1996; Messingham et al. 2001).

These findings are indicative of a risk for male patients with familial history of autoimmunity, and these patients may not be suitable for AD. The links between loss of testosterone and increased incidence of autoimmune disease in susceptible individuals are particularly pertinent given the huge interest in developing a milder preconditioning regime and faster immune regeneration so that HSCT can be safely used to treat severe autoimmunity. Any risks or benefits of loss of testosterone on the desirable graft-versus-autoimmunity effect are also yet to be assessed.

6 Androgen Blockade Compared with Other Methods of Thymic Regeneration: Risks and Advantages

Growth factors such as FGF7 (KGF), IL7, IGF-1 and Growth Hormone play an important role in thymic biology and, similarly to SSA, have been shown to improve immune reconstitution when administered to animals and humans (Min et al. 2002; Alpdogan et al. 2001; Napolitano et al. 2003; French et al. 2002; Fahy et al. 2003).

6.1 Keratinocyte Growth Factor (KGF)

KGF is produced by thymic fibroblasts as an essential growth factor for TEC development. The KGF receptor is expressed on all TEC subsets, but on a minority of cells by histology (Rossi et al. 2007b). When administered prior to BMT, KGF increased all donor-derived thymocyte subsets, regardless of the radiation dosage used to precondition the recipients (Min et al. 2002) and showed the added benefit of reducing the incidence of graft-versus-host disease (Rossi et al. 2002). The effect on the thymus appeared to be long-lived and resulted in increased peripheral T-cell function (Min et al. 2002). To date, there is no evidence that KGF is effective in humans, although primate studies appear extremely promising (Seggewiss et al. 2007).

Since KGF is not essential for castration-mediated thymic regrowth (Gabrielle Goldberg, unpublished observations), it is possible that coadministration of KGF and LHRH-A may result in a synergistic effect or widen the proportion of respondents for immune regeneration.

6.2 Growth Hormone

Growth hormone is a highly pleiotropic growth factor; which, in the thymus, acts indirectly on both thymocytes and TECs to stimulate proliferation (Mello-Coelho et al. 1998; Ferone et al. 1999) and in the bone marrow drives expansion of T- and B-cell progenitors (Knyszynski et al. 1992; Tian et al. 1998; Sumita et al. 2005).

Numerous studies have demonstrated its ability to induce thymus and bone marrow rejuvenation in aged animals and humans (Weigent, 1996; French et al. 2002; Fahy et al. 2003; LeRoith et al. 1996); however, the increased immune response never reaches normal young levels. After immunoablation and BMT, GH administration did, however, increase the numbers of bone marrow progenitors to normal young levels (Tian et al. 1998; Carlo-Stella et al. 2004), which translated to increased numbers of peripheral CD4 and CD8 T-cells and B-cells as well as increased thymus cellularity (Chen et al. 2003). Recombinant human GH has been assessed for its ability to improve immune function in HIV-infected patients, who showed significant increases in thymic mass and numbers of circulating naïve CD4+ and CD8+

T-cells (Napolitano et al. 2002; Pires et al. 2004). The dosage required to induce these effects (daily injections for one year) results in significant side effects such as myalgia, lethargy, headaches, and insulin insensitivity.

GH is likely to act at least partially through inducing transcription of IGF-I, which, when administered alone, is able to increase the proportion and number of T- and B-cells after immunoablation (Napolitano et al. 2003). Both GH and IGF-I treatment were shown to alter CD4:CD8 ratios in various organs in rhesus monkeys (LeRoith et al. 1996), with unknown long-term effect.

GH and sex steroid feedback pathways intersect (Chowen et al. 2004) such that serum GH levels are increased after administration of testosterone and reduced after castration, complicating the potential to utilise both therapies together for a synergistic effect. However, preliminary studies from this laboratory show that castration increases thymic-derived GH (Maree Hammett, Ann Chidgey, unpublished observations), and a combination approach for GH treatment is attractive since it may allow a reduction in the necessary dose, which would reduce its side effects.

6.3 SSA: Risks and Sequelae

Although there is an extensive safety profile on LHRH usage, with no major clinical side effects, it is not without adverse effects and it does impact upon the quality of life for patients.

The early sex steroid flare which occurs following LHRH-A treatment is a significant side-effect which in prostate cancer patients can reportedly result in increased proliferation of cancer cells leading to pain, uremia, the development of neurologic sequelae, including paralysis, and very rarely, death (Thompson 2001). This flare can be entirely prevented by treating the patient with sex steroid receptor blockers, including flutamide and bicalutamide, beginning prior to treatment. Studies suggest this therapy should be considered for all prostate cancer patients treated with LHRH-A (reviewed by Thompson 2001) and presumably Tamoxifen treatment would abrogate similar risks for women. Treatment with an LHRH-A does not harm future reproductive function in women (Heger et al. 2006). However, osteopenia is a distinct risk in all patients, since estrogens in both males and females are involved in bone metabolism (Lupoli et al. 1997; Smith et al. 2003). Many therapies have been proposed to reduce the risk of osteoporosis (reviewed by Moyad 2002) including treatment with Tamoxifen and other estrogen-receptor binding agents, calcitonin and fluoride, as well as complementary treatments such as calcium and vitamin D supplements. Men treated with an androgen blocker do not show signs of impaired bone mineral density (Smith et al. 2003). Future development of therapies with fewer side effects require full understanding of the mechanisms involved in thymic involution and regeneration to perhaps develop an immune-specific regenerative therapy.

One risk inherent in removing any cell-suppressive factor is predisposition towards the development of cancer (in this case, thymoma or lymphoma). Oyan and colleagues (2004) linked the development of thymic cancer after treatment of pro-

gesterone-receptor positive breast cancer with the ER blocker Tamoxifen (as well as chemotherapy and radiotherapy), which is the first such published occurrence; the frequency of this effect remains to be determined. In the BUF/Mna rat model of thymoma, estrogen decreases tumour incidence despite genetic predisposition (Ezaki et al. 1992) suggesting that SSA in females may increase its incidence. Long-term studies following patients once treated with Tamoxifen (for example, Rutqvist et al. 2007) have not revealed increased thymoma incidence, but this may be due to insufficient statistical power. However, the possible increased risk, if any, of thymoma must be weighed against the increased risk of cancer developing due to the absence of tumour immunosurveillance in the immunodepleted patient. For example, mice with the severe combined immunodeficiency (SCID) mutation develop thymic lymphomas after exposure to low levels of ionising radiation (Fulop et al. 1990; Lieberman et al. 1992). It is also now interesting that with continual improvement of antiretroviral therapy, AIDS patients have extended lifespans, which has exposed a predisposition to cancer onset. The very likely explanation for this is their lack of T-cell based immunosurveillance and there is thus additional cause and urgency to restore thymic function in these patients.

Despite the sex steroid-dependent ability of LHRH-A treatment or castration to either increase or decrease autoimmune disease in mice and humans, there is no evidence of increased risk of autoimmunity in patients who have received LHRH-A treatment. However, this may be a pertinent consideration for patients with a strong family history of autoimmunity. Such results highlight the incredibly complex basis to autoimmunity and again demonstrate the care that will be necessary when formulating clinical trial protocols, such that inhibition of ongoing autoimmunity is matched with the need to restore immunity with an actively self-tolerant system.

With any form of treatment that serves to modulate the immune system it is vital to maintain the natural homeostatic balance of the various immune players or risk increased susceptibility to infection, cancer and autoimmune diseases, as well as hampering the effectiveness of the immunotherapy being used. This is an important benefit of SSA-mediated immune regeneration. It is also vital to weigh the potential risks and benefits to the target patient group. In over 2 decades, there have been no reports of increased, abnormal incidence of immunologically-based disease or cancer with LHRH-A use.

7 Summary and Conclusions

The immunosuppressive properties of sex steroids have long been reported. While peaks in corticosteroid production are invariably transient, sex steroid production occurs throughout life, thus driving progressive thymic atrophy and a decline in immune function.

Sex steroid ablation increases thymus size and function in both mice and humans, resulting in the export of self-tolerant, antigen-reactive naïve T-cells. Both mice and humans treated with an LHRH-A exhibit faster recovery from immunodepletion,

reducing the risk of death from life-threatening infection. SSA improves immune function through direct effects on peripheral T-cells, and indirectly by increasing thymic output. The naïve T-cells produced can replace oligoclonal memory T-cell clones which can often be defective or of irrelevant specificity.

The improvement in thymic, bone-marrow and immune system function induced by temporary LHRH-A treatment have been studied long-term, and appear to impose no immune dysregulation. This treatment shows very little risk compared to other immunomodulatory therapies, and side-effects such as the early flare in sex steroids are generally manageable with combination sex steroid receptor blockade treatment.

The development of an immune-regenerative therapy is of paramount clinical importance, given the number of patients with acquired immunodeficiencies. Reversible sex steroid ablation therapy promises to reversibly restore immune competence faster, through safely and quickly rejuvenating the thymus. When combined with other adjuvants, this therapy is likely to provide a new paradigm for the treatment of T-cell-based disorders.

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Nutraceuticals and Immune Restoration in the Elderly

Barry W. Ritz and Elizabeth M. Gardner

Abstract: Nutraceuticals, including dietary supplements and functional foods, are a $152 billion world market. The percentage of those aged 65 years and older using nutraceutical products is higher than for any other age group and has doubled in recent years. Aging is associated with decreased immunity, increased morbidity and mortality resulting from infectious agents, and poor nutritional status. Deficiencies in vitamin E, vitamin B_6, folate, zinc, and selenium, for example, are particularly common, and deficits in these micronutrients have been reported to negatively influence immunity. Thus, if nutraceutical products can improve micronutrient status, the regular use of nutraceuticals by the elderly population may provide an opportunity to enhance immunity in this at-risk population. Results from human clinical trials evaluating the use of nutraceuticals to support immune restoration in the elderly, however, have been largely inconsistent. Additional clinical trials using consistent outcome measures are needed, which will require a cooperative commitment from the nutraceutical industry and academia.

Keywords: Elderly • Nutraceutical • Dietary • Supplement • Immune • Multivitamin

B. W. Ritz (✉)
Drexel University
Department of Bioscience & Biotechnology
Philadelphia, PA 19104, USA
Tel: +215-895-1435
Fax: +215-895-1273
E-mail: ritz@drexel.edu

E. M. Gardner
Department of Food Science and Human Nutrition
Michigan State University
East Lansing, MI 48824, USA

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1 Introduction

It has been postulated and highly substantiated throughout this text that decreased immunity is at least partially responsible for the observed increase in morbidity and mortality resulting from infectious agents in the elderly. However, it has also been emphasized that the effects of aging on immunity are highly heterogeneous, including among the healthy elderly, suggesting that additional factors might influence susceptibility to infectious disease in the aged. Nutritional status has been proposed as one such variable that may explain differences in both the incidence and the pathology of infection. For example, the elderly are at an increased risk for micronutrient deficiencies due to a variety of factors, including social, physical, economic, and emotional obstacles to eating [20, 41, 50]. Deficiencies in vitamin E, vitamin B_6, folate, zinc, and selenium are common in the elderly and deficits in these micronutrients have been reported to negatively influence immunity [1, 5, 44, 50]. Supplementation with these and other nutrients as potential nutraceuticals to restore immunity in the elderly will be discussed in detail in this chapter.

2 Nutraceuticals

The term ***nutraceutical*** encompasses a broad spectrum of commercially-available products in which a food or part of a food (nutrient) is intended to provide medical or health benefits, including the prevention and treatment of disease (pharmaceutical) [17, 18]. Nutraceuticals have no formal regulatory definition, so a variety of alternative definitions have emerged that largely debate whether the nutrient or pharmaceutical characteristics of nutraceuticals should be emphasized. Here, we broadly define nutraceuticals to include functional foods, dietary supplements, and medical foods (Table 1). Like nutraceuticals, ***functional foods*** have no legal definition, but are generally distinguishable from other types of nutraceuticals, because they are recognizable as conventional food products. In contrast, ***dietary supplements*** were legally defined in the Dietary Supplement Health and Education Act (DSHEA) of 1994, which expressly states, among other requirements, that products labeled as dietary supplements may not be represented as conventional foodstuffs [69]. A final category, ***medical foods***, is distinguishable from functional foods and dietary supplements by the requirement that medical foods meet "distinctive nutritional requirements of a disease or condition." [69] Defined according to the U.S. Orphan Drug Act, medical foods must be a food for oral or tube feeding, be labeled for the dietary management of a specific medical disorder, disease, or condition for which there are established nutritional requirements, and be intended for use under medical supervision. However, like all foods and dietary supplements, medical foods do not require premarket approval or registration with the United States Food and Drug

Table 1 Distinguishing characteristics of nutraceuticals and related product categories

Term	Definition	Appearance	Examples
Nutraceuticals	Any substance that may be considered a food or part of a food that provides medical or health benefits, including the prevention and treatment of disease. (DeFelice, DeFelice)	Foods, pills, tablets, capsules, powders, meal plans	Functional foods, dietary supplements, medical foods, genetically engineered foods
Functional foods	Those foods that encompass potentially healthful products, including any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains. (Milner, Thomas)	Foods	Fortified breads and cereals, calcium-enriched orange juice, energy drinks, energy bars
Dietary supplements	Any products which contain one or more dietary ingredients, such as vitamins, minerals, herbs or other botanicals, amino acids, or other ingredients used to supplement the diet. (CFSAN)	Pills, tablets, capsules, liquids, powders	Multivitamins, multiminerals, isolated nutrients, functional foods labeled as dietary supplements (e.g., bars)
Medical foods	A food which is formulated to be consumed or administered enterally under the supervision of a physician and for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation. (CFSAN)	Liquids or powders	Nutrient-modified products for patients with kidney/renal disease, diabetes, AIDS, cancer, cystic fibrosis, malabsorption, or metabolic disorders (phenylketonuria); oral rehydration solutions

Administration (FDA). The regulation of nutraceutical products is described in more detail at the end of this chapter.

3 Aging, Malnutrition, and Micronutrient Deficiency

As summarized in Table 2, the effects of aging, malnutrition, and micronutrient deficiencies on various parameters of immunity are complementary, if not cumulative [40, 60, 64]. As in aging, malnutrition and nutrient deficiencies are generally characterized by decreased delayed-type hypersensitivity (DTH) reactions, lymphopenia, reduced lymphocyte proliferation in response to mitogenic or antigenic stimulation, altered cytokine production, decreased antibody response to vaccination, reduced cytotoxic T lymphocyte (CTL) activity, impaired phagocytic function, and a loss in inducible natural killer (NK) cell activity. Thus, nutritional status and advanced age are independent but confounding factors that both have primary effects on innate and cell-mediated immunity, increasing the risk of infection.

Table 2 Reported effects of aging, malnutrition, and micronutrient deficiency on immune function[a,b]

Parameter	Aging	Malnutrition	Deficiency
Lifespan	NA	↓	↓
Infection	↑	↑	↑
DTH reactions	↓	↓	↓
Lymphocyte number or percentage	↓ or =	↓	↓
Lymphocyte proliferation	↓	↓	↓
Cytokine production	Altered (↓ IL-2)	Altered (↓ IL-2)	Altered (↓ IL-2)
Antibody response to vaccination	↓	↓	↓
CTL activity	↓	↓	↓
Phagocytic function	↓	↓	↓
Inducible NK cell activity	↓ or =	↓	↓

[a] Adapted from [60], used with permission
[b] Reported in animal studies and/or human trials

4 Nutritional Status in the Elderly

In controlled experimental conditions, nearly any nutritional deficiency, if severe enough, will result in impaired immunity and an increase in infectious disease; likewise, addressing the specific nutrient deficiency will restore immunity. This was elegantly demonstrated in a study by Meydani et al. [46] in which healthy elderly subjects were depleted of vitamin B_6 over a period of under 3 weeks. Vitamin B_6 repletion was then initiated in three, 3-week periods of increasing B_6 intake, followed by 4 days of supplementation at 50 mg/d. Vitamin B_6 depletion resulted in a decreased percentage and number of total lymphocytes, reduced lymphocyte proliferation in response to both B- and T-cell mitogens, and decreased IL-2 production. Following repletion, all parameters returned to baseline levels. Further, the authors demonstrated a significant correlation between plasma vitamin B_6 and mitogen-stimulated lymphocyte proliferation.

It is much more difficult to ascertain the effectiveness of nutritional supplementation on immune restoration among the highly heterogeneous, healthy elderly. First, while it is generally accepted that nutritional deficiencies increase with age, not all studies that have investigated the effects of micronutrient supplementation on immune parameters in the elderly have reported decreased micronutrient status at baseline [2, 6, 31]. Further, while some studies have demonstrated an association between decreased micronutrient status in the elderly and impaired immunity, such as decreased mitogen-stimulated lymphocyte proliferation [51] or a reduced protective antibody response to influenza vaccination [25, 33], other studies disagree [26, 30]. For example, in a study of 61 ambulatory elderly with a mean age of 81 years, 54 % of elderly participants regularly consumed micronutrient supplements compared to 33 % of young controls [26]. Elderly participants had plasma concentrations of beta-carotene, retinol, alpha-tocopherol, and zinc that were equal to or greater than those of young. However, despite adequate micronutrient

Table 3 Dietary reference intakes (DRIs) for men and women aged 70 years[a]

	Vitamin A (μg/d)	Vitamin C (mg/d)	Vitamin D (μg/d)	Vitamin E (mg/d)	Vitamin K (μg/d)	Thiamin (mg/d)	Riboflavin (mg/d)
Men	900	90	15	15	120	1.2	1.3
Women	700	75	15	15	90	1.1	1.1
UL[b]	3,000	2,000	50	1,000	ND[c]	ND	ND
	Niacin (mg/d)	Vitamin B_6 (mg/d)	Folate (μg/d)	Vitamin B_{12}(μg/d)	Pantothenic Acid (mg/d)	Biotin (μg/d)	Choline (μg/d)
Men	16	1.7	400	2.4	5	30	550
Women	14	1.5	400	2.4	5	30	425
UL	35	100	1,000	ND	ND	ND	3.5 g/d
	Calcium (mg/d)	Chromium (μg/d)	Copper (μg/d)	Iodine (μg/d)	Iron (mg/d)	Magnesium (mg/d)	Manganese (mg/d)
Men	1,200	30	900	150	8	420	2.3
Women	1,200	20	900	150	8	320	1.8
UL	2.5 g/d	ND	10,000	1,100	45	350	11
	Molybdenum (μg/d)	Phosphorus (mg/d)	Selenium (μg/d)	Zinc (mg/d)	Potassium (g/d)	Sodium (g/d)	Chloride (g/d)
Men	45	700	55	11	4.7	1.2	1.8
Women	45	700	55	8	4.7	1.2	1.8
UL	2,000	3 g/d	400	40	ND	2.3	3.6

[a] These values are set forth by the Food and Nutrition Board, Institute of Medicine, National Academies of Science; original reports available at www.nap.edu. These values represent a combination of Recommended Dietary Allowances (RDAs) and Adequate Intakes (AIs). The RDA is the average daily intake that will meet the requirements of nearly all (97–98 %) healthy individuals in the group. The AI is similar but is based on a limited amount of data, such that an RDA cannot be calculated. An AI for fluoride has also been determined for elderly men (4 mg/d) and women (3 mg/d). Fluoride is rarely found in multivitamin/multimineral products

[b] The Tolerable Upper Intake Level (UL) is the highest level of daily intake that is likely to pose no health risk to almost all individuals in the group. Additional ULs have been determined for boron (20 mg/d), fluoride (10 mg/d), nickel (1 mg/d), and vanadium (1.8 mg/d). The elderly should be cautioned against intakes above the DRI when a UL has not been determined

[c] ND, not determined

status, mitogen-stimulated lymphocyte proliferation was significantly reduced in the elderly participants. Therefore, it appears that some degree of immunosenescence occurs in the healthy elderly, independent of nutritional status.

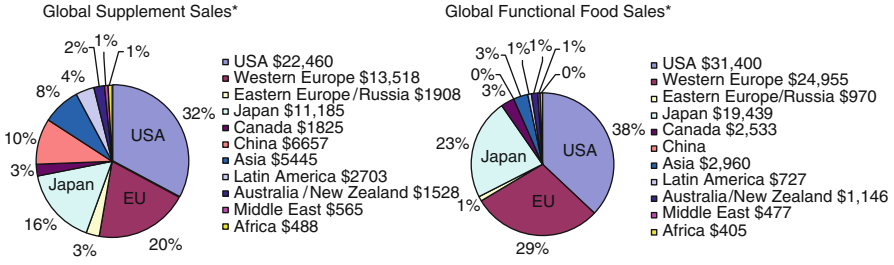
Specific micronutrient intake requirements have been established for elderly men and women by the Food and Nutrition Board, Institute of Medicine, National Academies of Science (Table 3). These Dietary Reference Intakes (DRIs) are intended to meet the daily needs of most healthy men and women aged 70 years or greater and serve as a reference for this population in both the US and Canada. In the elderly with low micronutrient status, supplementation with a multivitamin/multimineral at or near the DRI can successfully increase serum concentrations to target levels [7, 43].

5 Nutraceutical Use in the Elderly

Dietary supplements and functional foods are a $152 billion world market (Fig. 1) [54]. According to the *Nutrition Business Journal*, dietary supplement sales in the U.S. alone account for an estimated $22.5 billion and growing, including $7.5 billion in vitamin products and $4.6 billion in herbals (2006 data). Dietary supplement use is increasing among the nation's elderly and may provide an opportunity to correct nutrient deficiencies in this at-risk population. The percentage of those over the age of 65 using dietary supplements is higher than for any other age group in the U.S. and has nearly doubled in recent years [13, 36]. Although estimates vary by study criteria, it is clear from multiple reports that over 50% of those over the age of 65 currently use dietary supplements [3, 13, 26, 38, 53].

In a large, cross-sectional study of free-living adults across 13 states, dietary supplement use significantly increased with age and was positively associated with other healthy behaviors [3]. Dietary supplement use is generally higher in women, and use appears to peak in both men and women at ages 71–75 years, in terms of both the total percentage of the population utilizing supplements and total supplement intake [38, 66]. Multiple surveys have concluded that the nutraceuticals most frequently consumed by the elderly are multivitamins, followed by vitamins E and C [23, 35, 38]. Calcium supplementation is also high, especially in women, and is increasing [35, 38]. According to one recent study, approximately 23% of elderly men and 26% of elderly women report current use of herbal products [36].

The majority of nutraceuticals used by the elderly include dietary supplements in the form of multivitamins/multiminerals or individual vitamins and minerals, such as vitamin A, beta-carotene, vitamin B_6, vitamin E, and zinc. Studies supporting and detracting from their use in restoring immunity are summarized in Table 4 and discussed below. The use of probiotics is also discussed. Some traditional-use herbal/botanical products and specialty nutraceutical products, such as mushroom preparations, have demonstrated promising immunomodulatory effects in in vitro and animal studies, but clinical trials in the healthy elderly are unavailable.



*Sales in Millions of US Dollars
Data from *Nutrition Business Journal*, used with permission.

Table 4 Clinical trials evaluating the effects of nutraceuticals on parameters of immunity in the elderly

References	Age	*N*	Duration	Form, dose/d	Results
Multivitamin/multimineral					
[8]	59–85	56	1-year	Multi or multi + 100 mg/d Zn	↑ DTH (higher in multi group without Zn) ↑ lymphocyte proliferation ↑ NK cell activity
[56]	83	30	28 days	8,000 IU vitamin A, 50 mg vitamin E, 100 mg vitamin C	↑ lymphocyte proliferation ↑ T-cell number
[7]	59–85	56	1-year	Multi	↑ DTH
[29]	65–102	81	2 years	20 mg Zn, 100 μg Se or 20 mg Zn, 100 μg Se, 6 mg beta-carotene, 120 mg vitamin C, 15 mg vitamin E	↓ respiratory and urogenital infections
[10]	60–89	72	10 weeks	Multi	↑ DTH = lymphocyte proliferation = lymphocyte number
[6]	78	31 f	10 weeks	Multi	= lymphocyte proliferation = lymphocyte number
[43]	50–87	80	8 weeks	Multi	↑ micronutrient status = IL-2, 6, 10 production = PGE_2
[31]	≥60	652	15 months	Multi	= rate of infection
[4]	45–64 or ≥65	130	1-year	Multi	↓ rate of infection ↓ work absenteeism *Type 2 diabetics, high prevalence of subclinical micronutrient deficiency
[39]	≥65	34	183 days	Multi (8oz/d liquid)	↓ upper respiratory tract infections (days of symptoms) ↑ antibody response ↑ lymphocyte proliferation
[2]	65	910	1-year	Multi	= rate of infection
[42]	85	748	18 months	Multi	= rate of infection overall ↓ rate of infection in nondemented elderly *Institutionalized, high prevalence of micronutrient deficiency
Vitamin A					
[52]	76	129	90 days eval., 4,498 days follow up	200,000 IU (60,000 μg RE) + 40 IU vitamin E (single dose)	= bacterial infections *Institutionalized, low prevalence of suboptimal vitamin A status **High dose supplement did not result in toxicity

Table 4 (continued)

References	Age	*N*	Duration	Form, dose/d	Results
[24]	80	118	3 months	800 μg	= lymphocyte proliferation ↓ CD3+, CD4+ T cells
Beta-carotene					
[71]	56	20	2 months	30 or 40 mg	↑ CD4+ T cell percentage ↑ NK cell percentage ↑ IL-2R expression
[61]	51–64 or 65–86	59 m	10–12 years	50 mg (alternate days)	↑ NK cell activity = NK cell percentage = IL-2 production
[62]	60–80	23 f	3 weeks	90 mg	= DTH = lymphocyte proliferation = IL-2, PGE_2 production
[62]	50–86	54 m	10–12 years	50 mg (alternate days)	= DTH = lymphocyte proliferation = IL-2, PGE_2 production
[61]	65–88	34 m	12 years	50 mg (alternate days)	↑ NK cell activity = NK cell percentage = IL-12, IFN-α, IFN-γ
Vitamin B6					
[68]	65–81	15(14 m)	2 months	50 mg	↑ lymphocyte proliferation
[46]	64	8	4 days (after 3 weeks depletion)	50 mg	↑ lymphocyte proliferation ↑ lymphocyte number ↑ IL-2 production
Vitamin C					
[14]	≥65	15	3 weeks	2 g	= DTH = lymphocyte proliferation *Most subjects on cardiovascular medications
Vitamin E					
[47]	≥65	32	30 days	800 mg	↑ DTH ↑ lymphocyte proliferation ↑ IL-2 production ↓ PGE_2 production
[45]	≥65	88	235 days	200 mg	↑ DTH ↑ antibody response
				800 mg	↑ antibody response *60mg = no effects
[19]	67–85	83	3 months	100 mg	= lymphocyte proliferation = IgG and IgA levels
[15]	72	30 f	16 weeks	200 mg + 1 g vitamin C	↑ lymphocyte proliferation ↑ phagocytic function ↓ lipid peroxidation ↓ cortisol
[55]	65–80	161	6 months	100 mg	↑ DTH= lymphocyte proliferation = IL-2, IL-4, IFN-γ *50 mg = no effects

Table 4 (continued)

References	Age	*N*	Duration	Form, dose/d	Results
[16]	≥60	37 m	3 months	200 mg	↑ lymphocyte proliferation
[34]	50–69	21,796 m	4 years	50 mg	5 % ↓ in the incidence of common colds (nonsmokers)*No association with vitamin C or beta-carotene
Meydani 2004	≥65	617	1-year	200 mg	↓ upper respiratory tract infections
[72]	≥	40	3 months	200 mg + 5 g fish oil	= DTH = lymphocyte proliferation *Increases in plasma level of α-tocopherol and T cell function may have been blunted by fish oil intake
Zinc					
[21]	81	30	1 mo	220 mg $ZnSO_4$	↑ DTH ↑ T cell number ↑ IgG antibody response = lymphocyte proliferation = lymphocyte number *Institutionalized, healthy elderly, no baseline Zn status reported
[12]	65–78	8 m	4.5 months	60 mg	↑ DTH = lymphocyte number *Zn-deficient at baseline
[57]	50–80	13	6 months	30 mg	↑ DTH ↑ IL-1 ↑ plasma thymus hormone activity *Zn-deficient at baseline
[24]	80	118	3 months	25 mg $ZnSO_4$	↑ CD4+ T cell number ↑ CTL number ↓ lipid peroxidation
[59]	64–100	384	60 days	400 mg	= antibody response
[28]	65–103	725	2 years	20 mg Zn +100 μg Se	= DTH ↑ antibody response ↓ respiratory infection (p=0.06)
[58]	55–87	50	12 months	45 mg	↓ rate of infection *Elderly had lower Zn status at baseline than young controls
Probiotics					
[27]	63–84	30	3 weeks	*Bifidobacterium lactis* HN019 (5×10^{10} CFU)	↑ CD4+ and CD25+ T cell percentage ↑ NK cell percentage ↑ NK cell activity ↑ phagocytic function
[65]	44–80	52	3 weeks	*Lactobacillus rhamnosus* HN001 (2.5×10^{10} CFU)	↑ NK cell activity ↑ phagocytic function

6 "Multis"

Results of randomized controlled trials on the effects of multivitamin/multimineral supplements on immune outcomes in the elderly have been greatly inconsistent. Multiple reports have shown no benefits [2, 6, 31, 43], while others have demonstrated positive effects on immunity, including decreased rates of infection, in specific elderly populations, such as the nondemented, institutionalized elderly [42] and those with comorbidities and confirmed micronutrient deficiencies [4]. The two largest randomized, double-blind, placebo-controlled trials investigating the effects of multivitamin/multimineral supplementation on rates of infection in free-living elderly showed no effect on the incidence or severity of infection [2, 31]. Notably, these two studies enrolled healthy, mostly free-living elderly who were reported to be well-nourished.

7 Vitamin A and Beta-Carotene

Although the role of vitamin A in immunity has been well-studied in malnourished children and, more recently, in HIV+ adults [70], few studies have examined vitamin A as a potential nutraceutical for immune restoration in the elderly. In a double-blind, placebo-controlled trial in 129 institutionalized elderly, a single dose of vitamin A (200,000 IU) did not reduce the incidence of bacterial infections, as assessed over an initial evaluation period of 90 days and a total follow up of 4,498 days [52]. Notably, only approximately 12 % of the study population was deficient in vitamin A at baseline. Further, this single, high dose of vitamin A did not result in toxicity symptoms. Animal studies suggest that excess vitamin A suppresses both humoral and cell-mediated immunity [70].

Beta-carotene is a plant-derived carotenoid that serves as a provitamin, converted in vivo to vitamin A. While supplementation of the elderly has resulted in no consistent influence on T-cell-mediated immunity [62, 71], beta-carotene supplementation may mediate the age-associated decline in NK cell activity [61, 63]. It has been suggested that these effects might be better aligned to beta-carotene's role as a source of antioxidant-rich carotenoids than as a precursor to vitamin A. Studies evaluating clinical outcomes in the elderly, i.e., rates of infection, are not available.

8 B Vitamins

Deficiencies in vitamins B_6, B_{12}, and folate are common among the elderly, yet clinical trials to assess the direct effects of B vitamin supplementation on immune status are virtually nonexistent. In one study of 65 healthy, free-living men and women with a mean age of 70 years, serum vitamin B_6 status was positively associated with IL-2R expression, and both vitamin B_6 and folate were associated with

NK cell number [37]. In the same study, vitamins A, C, E, beta-carotene, and zinc were not associated with these parameters. It should be noted that most elderly supplement users will obtain B vitamins as part of a multivitamin.

9 Vitamin E

Vitamin E status has been negatively related to rates of infection in the elderly [11], and numerous clinical trials have demonstrated positive effects of vitamin E supplementation on immunity in the healthy elderly [16, 34, 45, 47, 48, 55]. Reported benefits include increased DTH reactions, increased lymphocyte proliferation, increased antibody response to vaccination, and decreased rates of infection. Animal data suggest that vitamin E may mitigate age-related changes in the plasma membrane, as well as gene expression associated with T-cell survival, transcriptional regulation, signal transduction, and cytokine production [72]. Not all clinical trials have been positive, however. In a large randomized, double-blind, placebo-controlled, 2 × 2 factorial study of 652 community-dwelling elders aged 60 years or older, supplementation with 200 mg of vitamin E demonstrated no effect on the incidence of acute respiratory tract infections [31]. Instead, supplementation was associated with a small but significant increase in the severity of infections.

10 Zinc

A number of studies have documented poor zinc status in the elderly and improved immunity upon supplementation [12, 57, 58]. A randomized, double-blind, placebo-controlled trial of 50 healthy elderly subjects aged 55–87 years and representative of multiple ethnic groups revealed a low plasma zinc status at baseline and a significant reduction in total infections when supplemented with 45 mg of elemental zinc as zinc gluconate over a period of 1-year [58].

11 Probiotics

Probiotics are nonpathogenic microorganisms commonly consumed in fermented dairy products, such as yogurt or kefir. Probiotic research has primarily focused on their positive effects on digestion, as well as potential therapeutic applications in the treatment of diarrhea and gastrointestinal disorders. However, results from animal studies have demonstrated positive effects on immunity, as well [9]. Two small human clinical trials suggest a potential for various probiotic strains to increase NK cell activity and phagocytic function in the elderly [27, 65].

12 Regulation of Nutraceutical Products

Across the world, governments differ in their definition and regulation of nutraceutical products. Although nutraceuticals are not regulated like pharmaceuticals in the U.S. or Canada, they are indeed regulated. The FDA and Health Canada are principally responsible for the regulation of nutraceutical products in the U.S. and Canada, respectively, and these agencies appear to collaborate. In the U.S., nutraceuticals and functional foods have no legal definitions and, therefore, must be placed in one of a number of existing categories, including conventional foods, food additives, dietary supplements, medical foods, or foods for special dietary use (infant formulas or hypo-allergenic foods). Most nutraceutical products used by the elderly are dietary supplements, which are regulated as a subcategory of foods, not as drugs. Unlike drugs, dietary supplements composed of ingredients in use prior to the passage of DSHEA in 1994 do not require premarket approval by FDA. New dietary ingredients, however, must be the subject of a 75-day premarket notification submitted to FDA before use. Under DSHEA, dietary supplement manufacturers are responsible for ensuring that label information is truthful and not misleading and that a product is safe before it is marketed. The FDA is then responsible for taking action against any unsafe or mislabeled dietary supplement after it reaches the market. While FDA oversees dietary supplement labeling and the proper use of label claims (**See Inset**), the Federal Trade Commission (FTC) regulates truth in dietary supplement advertising. Thus, while dietary supplements do not require efficacy studies prior to going to market, manufacturers making claims about a product's health benefits may be asked for substantiation by federal or state agencies.

As part of DSHEA, Congress authorized FDA to establish current good manufacturing practices (CGMPs) for dietary supplements. In June of 2007, FDA issued its final rule to:

(1) require standards in manufacturing, packaging, labeling, and holding of dietary supplements to ensure that a dietary supplement contains what it is labeled to contain and is not contaminated with harmful or undesirable substances, such as pesticides, heavy metals, or other impurities, and,

Nutraceutical products labeled as dietary supplements may bear label claims that describe the role of a nutrient or dietary ingredient intended to affect the structure or function of the body. Examples include "calcium builds strong bones" or "antioxidants maintain lymphocyte function." The legal use of such *structure/function claims* was set forth by DSHEA. These claims utilize language distinctly different from drug or disease claims and must be accompanied by the following disclaimer: "These products have not been evaluated by the FDA. These products are not intended to diagnose, cure, mitigate, treat, or prevent any disease."

(2) require certain activities that will ensure the identity, purity, quality, strength, and composition of dietary supplements, which is a significant step in assuring consumers they are purchasing the type and amount of ingredients declared [69].

These regulations will be fully implemented by 2010.

13 Safety of Nutraceutical Use in the Elderly

According to Dr. Pamela Haines at the Department of Nutrition, School of Public Health, University of North Carolina at Chapel Hill, "elderly supplement users appear to fall into four categories: (1) those who are dissatisfied with current medical care, (2) those who prefer to follow the growing movement for health promotion and greater selfcare, (3) those treating either real or perceived symptoms of aging, and (4) those with chronic diseases." [13] Overall, the elderly appear to consume vitamins and other nutraceutical products for their perceived health benefits, including the restoration of immunity. Notably, in one recent study, only about 5–6 % of the elderly reported using nutraceutical products at the recommendation of a physician [36].

Without adequate oversight by health care practitioners, the safe use of nutraceuticals by the elder consumer remains a concern. It is important to recall, as noted at the beginning of this chapter, that the elderly are a highly heterogeneous group. At the *Conference on Dietary Supplement Use in the Elderly* held at the NIH in January, 2003, Dr. Tamara Harris noted in her comments that "while the elderly are often grouped into one category, in reality they present on a continuum from healthy to frail." [13] Thus, additional data are needed to define nutritional requirements in the diverse elderly, including the determination of how micronutrient requirements change with age, how comorbidities associated with advanced age affect micronutrient status, and which subpopulations of the elderly would most benefit from nutritional interventions. Further, the altered absorption, metabolism, and excretion of nutraceutical products in this diverse age group must be considered. Finally, increased age may elevate the potential for drug-nutrient interactions due to physiological changes that occur with age, altered drug metabolism, or an increase in polypharmacy (concurrent use of multiple prescription drugs) [13, 32].

14 Summary

Nutraceuticals empower the elderly individual to make personalized health care decisions believed to promote healthy aging and provide a practical means to meet micronutrient requirements. The efficacy of nutraceuticals to promote immune restoration requires further study, however, as some clinical trials support the use of multivitamins/multiminerals, individual nutrients, like vitamin E, and specialty products, while other studies show no benefits or, in rare instances, harm. Further, despite an increased risk of micronutrient deficiencies, the routine use of multi-

vitamin/multimineral products by the healthy elderly is not recommended at this time, pending additional clinical trials using consistent outcome measures [22, 67]. Such studies will require a cooperative commitment from industry and academia. Nonetheless, the reality is that the majority of those over the age of 65 already use nutraceutical products regularly, and rates of usage continue to rise. Although nutraceuticals are indeed regulated, the safety of nutraceutical products is determined by the manufacturer and nutraceuticals do not have to be proven efficacious prior to sale. Therefore, elderly consumers should exercise caution in choosing appropriate nutraceutical products.

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Gene Therapy and Immune Senescence

Jian Chen, Hui-Chen Hsu and John D. Mountz

Contents

1 Principles of Gene Therapy

Gene therapy can be classified according to the vector used for gene therapy and the transgene that will be expressed as a result of the gene therapy. One consideration for gene therapy is that certain vectors have larger capacities than others to incorporate genes. The second consideration is the duration of therapy, which depends upon the immune response after delivery of the therapy. Therapy duration usually exhibits a reciprocal relationship to therapy immunogenicity. A third consideration is safety. This is related both to the immunogenicity and adverse effects of potential integration. These factors are shown in Table 1. Advances to enable gene integration with safety have been carried out using a "suicide" gene, such as thymidine kinase (TK) that can be upregulated to eliminate cells in which the transgene has integrated into an adverse position in the genome.

J. D. Mountz (✉) · J. Chen · H.-C. Hsu
Birmingham VA Medical Center, 1825 University Blvd
SHEL no B310, The University of Alabama at Birmingham
Birmingham, Alabama 35294, USA
Tel: (205) 934-8909
Fax: (205) 996-6788
E-mail: john.mountz@ccc.uab.edu

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Table 1 Gene therapy delivery vectors applicable for immune senescence reversal

Viral vector	Vector capacity	Immunogenicity	Therapy duration	Safety and gene integration
Adenovirus	36 kb	High	Weeks	Toxic immune response
Adeno-associated virus	6.5 kb	Medium	Months	Highest safety
Retrovirus	10 kb	Low	Years	Integration risk

1.1 Gene Therapy Production and Delivery Considerations; Lesions from Human Trials

1.1.1 Gene Therapy with Adenovirus to Correct Immune Senescence

Since 1990, Adenovirus (Ad) vectors have been the vectors of choice for gene therapy application because of many favorable features [1]. These features include the ability to grow recombinant viruses to high titers, a relatively high capacity for transgene insertion, and efficient transduction of both quiescent and actively dividing cells, usually without incorporation of viral DNA into the host cell genome. However, its application is limited by the toxicity associated with the use of Ad vectors, which is complex involving both the innate and adaptive immune response [2, 3]. The initial response to Ad vectors administered intravascularly occurs within minutes, peaks at 6 hrs, and occurs in the absence of viral gene expression. This response has been attributed to the innate response characterized by the release and/or production of several proinflammatory cytokines including IL-6, TNF-α, IL-8, IL-12, IFN-γ, RANTES, and GM-CSF [4–6]. The cells that participate in the innate immune response are macrophages, dendritic cells (DCs), so-called professional antigen presenting cells (APCs), and also NK cells that serve as a functional bridge between the innate and acquired immune response. Immature APCs are activated, resulting in the upregulation of MHC antigens as well as costimulatory and adhesion molecules, via an NF-κB-dependent pathway [7, 8]. Mature DCs, loaded with antigenic peptides, migrate to draining lymph nodes, where they deliver "signal 1" to naïve T-cells through the interaction of peptide-MHC complex with T-cell receptor (TCR). B7-1/B7-2 (CD80/CD86) molecules on APC interact with CD28 on T-cells, delivering "signal 2," which is critical for cytotoxic T-lymphocyte (CTL) cross-priming by activated APC. In addition, vector interaction with epithelial cells results in the release of C-X-C chemokines, especially IP-10, which is a potent chemoattractant for activated T-lymphocytes and polarize the reaction towards a Th-1 type response [9, 10].

Generation of Ad viral antigen or transgene product-specific CTL response plays a major role in limiting transgene expression by eradiation of transduced cell [11, 12]. Previous studies of CTL response against Ad focused on the activation and the

effector function of CD8 T-cells. The importance of CD4 T-cell help for primary CD8 T-cell responses remains controversial. Early observation of *in vivo* ablation of $CD4^+$ cells or interferon IFN-γ was sufficient to prevent the elimination of Ad-transduced hepatocytes, despite the induction of a measurable CTL response prolonged indicated that CD4 T^+ cells may be necessary for a fully competent CTL response [13]. The presence of CD4 cells during the priming phase is critical for generating functional CD8 memory, and direct CD40-CD40L interaction between CD4 and CD8 cells might be involved in this process [14]. Further studies, however, have shown that the primary CTL response to infectious agents, including to Ad, is often independent of T_H [15, 16], and it was hypothesized that recognition of microbial products by Toll-like receptors can license DCs to prime an effective CTL and thus bypasses the need for CD4 help [15, 17].

Previous studies using replication-defective Ad (RDAd) indicated that FasL-Fas and TNF-TNFR pathways, but not perforin/granzymes pathways mediated the cytotoxic effector function of Ad specific CTL [18, 19]. However, direct analysis of the specific CTL response to Ad had been difficult due to the lack of reagents that recognize the rearranged TCR expressed on Ad-specific CTLs. To overcome this problem, we have recently generated an MHC class I tetramer and used an *in vivo* killing assay to enable direct quantitate the AdE1Bp-specific CTL response [3]. Our results reveal that during the primary response, there was a significant defect in both the generation and *in vivo* effector function of Ad-specific CTLs in $CD28^{-/-}$ mice, but not in $CD4^+$ T-cell-depleted mice or $CD4^{-/-}$ mice. The relative role of CTL effector molecules was assayed by an *in vivo* CTL assay in perforin- or FasL-mutant mice, using donor cells from Fas-deficient or TNFR1/TNFR2-deficient mice. The results indicated that the *in vivo* CTL activity is mediated mainly by perforin. In the absence of perforin, production of FasL, but not TNF-alpha, provided the major effector mechanism to induce Ad-specific killing of target cells [2].

We have applied the same strategy to determine the age-related changes in CTL to Ad in aged mice. Despite the immune response to Ad vectors, their use in older individuals may be safer than in younger individuals due to a decreased immune response. Administration of an AdE1b resulted in a lower E1b tetramer specific response in aged mice compared to young mice (Fig. 1). The adenovirus exhibits liver tropism, but encounters a lower immune response in the liver of aged mice. Thus adenovirus gene therapy is more feasible in aged animals, and possible in aged individuals, compared to younger animals.

1.1.2 Gene Therapy with AAV to Correct Immune Senescence

Recombinant adeno-associated virus (rAAV) vectors are unique among the vector classes currently available for human gene therapy in that they are based upon a class of viruses that commonly inhabits a human host without causing any detectable pathology. These vectors and their transgene product can be present for years after administration and may be the safest option for long term correction of immune senescence by supplementation with a cytokine or growth factor. Thus, in spite of

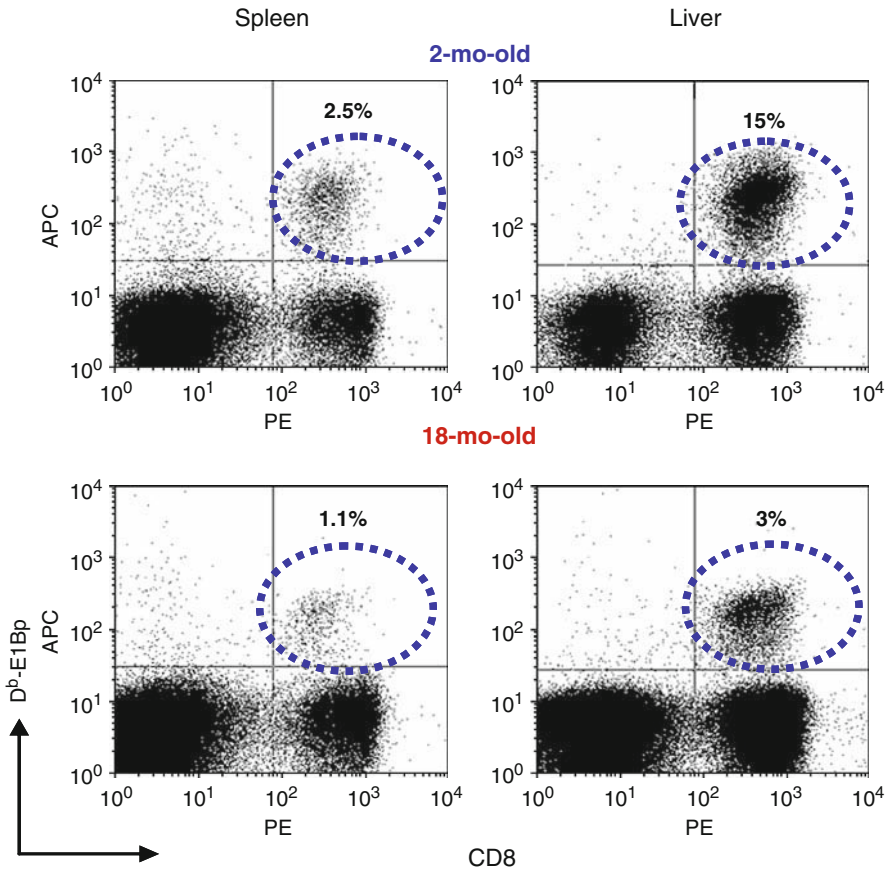

Fig. 1 Decreased E1b CTL response in aged mice. Young (2-month-old) and aged (15-month-old) B6 ($H2D^b$) mice were infected IV with wild type Ad5 (1×10^9 iu). The percentage of E1Bp specific CD8 T-cells in the spleen and liver of infected mice at day 8 was determined using an Ad E1Bp specific tetramer (circled population). There was a 58% reduction in the% of D^b-E1Bp specific CD8 T-cells from 18-month-old compared to that from 2-month-old mice, in both the spleen and liver (mean ± SEM; *N*=3 in each group). Adapted from the work of Chen et al. with permission from molecular Therapy (99)

their small capacity for the transgenes, there is a rapid increase in the study and application of AAV gene therapy. However, recent findings by us and others have indicated that AAV vectors cannot only induce strong humoral response marked by production of anti-AAV antibodies, but also induce a CTL response [20–23]. Together, these results indicate that AAV plus transgene can induce a potent CTL response that can lead to elimination of the vector and prevent transgene expression. Furthermore, this can occur after administration into nondendritic cells, including muscle cells, using a tissue-specific promoter. A recent fatality using AAV gene therapy in an immune suppressed patient with rheumatoid arthritis raises a possible warning that AAV gene therapy, in combination with other treatments, or in an immune compromised state such as ageing, may have unknown toxicities [24].

1.1.3 Gene Therapy with Retrovirus to Correct Immune Senescence

Correction of immune senescence, by definition, will require long term delivery of a therapeutic gene. Retroviral gene delivery offers the best option for long delivery of a gene therapy with minimal immune response leading to elimination of the gene therapy vector and its product. The risk of gene integration limits systemic use of retroviral vectors. However localized use of retroviral gene therapy has been successfully used in human to deliver anti-inflammatory gene for rheumatoid arthritis [25], and this is a feasible approach for potential correction of immune senescence.

2 Specific Transgene Expression and Promoter Considerations

Transgenes that are strong candidates for gene therapy to prevent cell senescence include transgenes for both maintenance of development or capacity of cells, and transgenes that can promote strong lymphocyte responses after activation. This chapter is largely devoted to discussion of potential transgene of gene therapy that can be used for reversal of immune senescence, and includes specific examples when these approaches have been successful, and when they have not been successful.

Transcriptional targeting facilitates spatially controlled, inducible, or physiologically regulated therapy by utilizing regulatory DNA sequences—promoters, enhancers, and/or silencers—to drive targeted expression of the therapeutic gene. Cell-specific promoters may be especially useful. T-cell specific promoters include the CD2 promoter, the Lck promoter and the CD4 promoter [26–28] (Table 2). For B-cells, a CD19 gene promoter in combination with a retroviral vector, or a lentiviral vector, in combination with an Ig kappa (Igk) light chain promoter and enhancer has previously been described as a useful B-cell-specific promoter in mice or humans [29–32]. Macrophage-specific promoters induce expression of cytokines and enzymes, include CD68 and c-fms [33, 34].

3 Stem Cells as a Potential Target for Age-Related Gene Therapy

Regenerative medicine endeavors to discover novel approaches to engineer through tissues of revitalized older tissues in an effort to “rejuvenate” failing and ageing components [35–41]. Kassem et al. [42] has recently reviewed that ageing is associated with the progressive failure of tissue and organs in the human body leading to a large number of age-related diseases. One of the most promising of future avenues is the use of embryonic and adult (somatic) stem cells. In this context, the use of gene therapy to modulate tissue differentiation or enhance the longevity or the replacement potential of such stem cells is receiving increased emphasis. This plays a role in many areas of human ageing including the heart,

Table 2 Lymphocyte and macrophage—specific promoter

Cell specific expression	Promoter	References
T-cells	CD2	[28]
	Lck	[27]
	CD4	[26]
B-cells	CD19 gene promoter in combination of a retroviral vector to express in mouse B-cells	[29]
	CD19 gene promoter in combination of a lentiviral vector to express in human B-cells	[31]
	Ig kappa (Igk) light chain promoter and enhancer	[30, 32]
Macrophage	Human CD68	[33]
	c-fms	[34]

lung, joint tissue including osteoblasts and osteoclasts, endothelial cells, neuronal cells, and immune cells.

With regard to immune cell and stem cell therapy, gene therapy has been used to modify $CD34^+$ stem cells. $CD34^+$ long-term repopulating hematopoietic stem cells can be employed to regenerate the hematopoietic system and therefore, lead to rejuvenation of the immune system. The question of whether hematopoietic stem cells age has raised considerable controversy, and has been re-opened recently, as a result of the growing interest in stem cells for transplantation and gene therapy [43]. Studies have focused on the generation of different blood cell elements and the capacity for selfrenewal; properties that characterize stem cells. Taken together, it appears that basal haematopoiesis is maintained throughout life, yet, the capacity to cope with hematological stress is decreased in advanced age. In principle, stem cells derived from aged donors can be used for autologous transplantation, when needed to recover basic haematopoiesis. However, patterns of T-cell development are altered in ageing, and intervention to augment T-cell response still needs to be considered. Current methods for expansion and maintenance of stem cells *in vitro* enable examination of stem cell potential for long-term expansion and function. A critical evaluation of the possible risks of replicative senescence and developmental changes in stem cells has become feasible. Ageing effects may relate to cell replication, cell migration and lymphoid differentiation. Understanding of the mechanisms underlying these processes will enable the fidelity of stem cell expansion and maintenance of their potential for long-term function.

Matsuoka et al. [44] have used the $CD34^+$ stem cells for potential long-term repopulation of bone marrow (BM) cells that then lead to further differentiation into the thymus and peripheral T- and B-cells. $CD34^+$ lin^- $C\text{-}kit^+$ cells have been studied by Gary Van Zant et al. [45–48] in the BXD recombinant inbred strain of mice. They have found that adult stem cells normally replenish tissue cells lost through the wear and tear of ageing or damage from injury or disease. With the proper coaxing in

tissue culture and when transplanted, these stem cells may regenerate the full repertoire of organotypic cells and thus may therapeutically regenerate tissues *in vivo* in much the same way as embryonic stem cells do. For several reasons, the best-studied stem cells are those of the blood-forming system. Mature blood cells generally have short functional life spans, usually measured in days, and therefore require replenishment at a steady pace throughout one's lifetime. Stem cells are intimately involved in this renewal and, because of the relative ease of access to the BM, stem cells have been well studied. Second, BM transplantation following radiation or high-dose chemotherapy in the treatment of cancer has fostered research on the basic biology and therapeutic uses of hematopoietic stem cells. Stem cells accumulate cellular damage during ageing that diminishes their developmental potency and ability to replenish blood cells, particularly after hematopoietic stress. In this view, the impaired function of stem cells in hematopoietic and in other self-renewing tissues limits the longevity of animals, and perhaps of humans. Identifying, and ultimately manipulating, the genes that regulate stem cell number, replication rate, and self-renewal capacity may have important clinical benefits.

Human umbilical cord blood-derived mesenchymal stem cells (UCBMSCs) are expected to serve as an excellent alternative to BM-derived human mesenchymal stem cells [49]. However, it is difficult to study them because of their limited life span. To overcome this problem, we attempted to produce a strain of UCBMSCs with a long life span and to investigate whether the strain could maintain phenotypes *in vitro*. UCBMSCs were infected with retrovirus carrying the human telomerase reverse transcriptase (hTERT) to prolong their life span. The UCBMSCs underwent 30 population doublings (PDs) and stopped dividing at PD 37. Whereas the UCBMSCs newly established with hTERT (UCBTERTs) proliferated for >120 PDs. The p16INK4a/RB braking pathway leading to senescence can be inhibited by introduction of Bmi-1, a polycomb-group gene, and human papillomavirus type 16 E7, but the extension of the life span of the UCBMSCs with hTERT did not require inhibition of the p16INK4a/RB pathway. The characteristics of the UCBTERTs remained unchanged during the prolongation of life span. Therefore UCBTERTs provide a powerful model for further study of cellular senescence and for future application to cell-based therapy by using umbilical cord blood cells.

With regard to gene therapy, Effros and Globerson have found that ageing hematopoietic stem cells play an important role in immune ageing [50]. This has been traced to defects in the development of T-cells that are altered with ageing, which may be related to replicative senescence as well as development of changes in stem cells. These can be reversed to some extent by gene therapy that can inhibit senescence by targeting telomerase and telomerase reverse transcriptase [51]. Therapeutic strategies for inhibiting telomerase activity have included both targeting components of telomerase (the protein component, TERT, or the RNA component, TERC) or by directly targeting telomere DNA structures. Recently a combination telomerase inhibition therapy has been studied also. The TERT promoter has been used to selectively express cytotoxic gene(s) in cancer cells and a TERT vaccine for immunization against telomerase has been tested.

4 Gene Therapy to Prevent Thymic Involution

Thymic involution begins in early adult life and leads to progressive loss of generation of naïve T-cells. This can be assessed by the thymocytes recombination incision circle (TREC). In this regard, Sempowski et al. [52] has shown that there is senescence of CD8 T-cells with decreased CD8 T-cell TREC before CD4 T-cells. However, the thymic involution has the most profound effect of ageing on the immune system resulting in a greatly decreased number of both CD4 and CD8 T-cell in the peripheral pool.

The effect of ageing on thymocyte progenitors in the BM was studied in an in vitro experimental model that permits T-lymphocyte development. The model is based on coculture of BM cells from young and old mice with lymphoid depleted fetal thymus explants. Globerson and coworkers [53] applied different strategies of thymic colonization, including competitive colonization by BM cells from different donor age groups and MHC backgrounds. Our data reveal intrinsic changes in the BM that lead to manifestation of immunosenescence in the T-lymphocyte compartment. Certain factors can be used to prevent thymic involution, including an extensively studied IL-7 [54] and long-term β-adrenergic receptor blockade [55]. These cytokines and growth factors affect thymocyte involution and T-cell development within the thymus. All of these may be potential targets for gene therapy.

4.1 Factors Associated with Thymic Involution

Factors related to thymic involution include soluble mediators are produced by thymic stromal cells, which are a source of a variety of growth, differentiation and survival factors. IL-7 has been shown to play a critical role in both mouse and human thymopoiesis, but the central role for a declining IL-7 and its contribution to thymic involution is controversial. Natural systemic factors that regulate thymopoiesis include changes in the endocrine system including sex steroids and growth hormone (GH) that are altered at puberty [56, 57]. Administration of testosterone or estrogen results in a decline and thymus size in experimental systems. As sex steroids rise at puberty, there is a decline in the production of GH, whose effect is mediated by local tissue induction of IGF1. The decreased production of GH and IGF1 during senescence contributes to thymic involution. Therefore, gene therapy targeted to these changes in the endocrine system may be valuable in preventing thymic involution or may play a role in the restoration of thymic function after involution.

Taub and Longo have extensively reviewed factors associated with thymus involution [58, 59]. With thymus involution, there is decreased expression of genes that are involved with thymus involution including leukemia inhibitor factor (LIF), as well as several thymus neurotropic family members, including TRKA and BDNF. Several neurotrophines, such as nerve growth factor (NGF) and brain-derived

neurotrophic factor (BDNF) decrease with age in the thymus. There are also changes in glucocorticoids which have both proapoptotic effects, as well as survival effects [60, 61]. Inflammatory cytokines are increased in older human thymus including IL-6, SEF, LIF, CNTF, and OSN. Reduced levels of zinc have been associated with thymocyte death. Supplementation of drinking water in aged mice with zinc sulfate has been reported to increase thymic mass and possibly thymopoiesis [62]. Age-related increase in adipocytes in the thymus is associated with thymic involution [63]. Adipocytes produce factors that reduce thymus size including IL-6, LIF, and leptin. Leptin further produces inflammatory cytokines including IL-6, IL-1β, TNFα, and IFN-γ. Adipogenic factors, such as LIF, insulin, glucocorticoids, and thyroid-stimulating hormone, are expressed at increased levels in the involuting thymus and can also induce fibroblasts to undergo adipocyte differentiation [64]. Therefore, further understanding of thymus involution is required to identify potential targets of gene therapy to prevent thymic involution.

By combining the mouse TREC assay with T-cell phenotypic analysis, we have demonstrated that rapid-involution strains of mice exhibited a developmental block at the DN1 to DN2 and $CD4^-CD8^-$ (DN) to $CD4^+CD8^+$ (double positive, DP) transition stages. There was also increased susceptibility to H_2O_2-induced apoptosis, decreased thymic expression of IL-7, decreased expression of an IL-7 receptor downstream anti-apoptosis gene, Bcl-2, and increased expression of a proapoptotic gene, Bad. In contrast, IL-7R expression was higher on DN thymocytes of rapid-involution strains. The increased expression of IL-7R was associated with an increased thymocyte proliferation in response to anti-CD3 + IL-7 or anti-CD3 + IL-12 + IL-7. IL-7 administration to young mice induced both increased thymopoiesis and peripheral T-cell proliferation [65].

Thoman et al has expressed IL-7 long term using the cell-gene therapy approach [66, 67]. Thoman has produced stromal cells that exhibit constitutive expression of IL-7 and has transplanted these stromal cells into the thymus. Increased local concentrations of IL-7 maintain the first stage of thymopoiesis and overcome their well-described block of DN1-to-DN2 transition. However, there is no decrease in thymic involution or increase in T-cell output. Therefore, these results suggest that in addition to prevention of the DN1/DN2 age-related transition block, blocks between other subsequent stages of double-negative $CD4^-CD8^-$ T-cell development in the thymus need to be corrected to prevent thymic involution. Gene therapy in other thymopoietic factors as described above may be promising in this regard.

In addition to IL-7, we have previously shown that IL-12 is another important cytokine that can maintain thymic integrity and function during the ageing process [68]. IL-12b knockout (*Il-12b*$^{-/-}$) mice exhibited accelerated thymic involution compared with wild-type (WT) B6 mice. This is characterized by an increase in thymocytes with the early development stage phenotype of $CD25^-CD44^+CD4^-CD8^-$ in aged *Il-12b*$^{-/-}$mice. Histologically, there were accelerated degeneration of thymic extracellular matrix and blood vessels, a significantly decreased thymic cortex/medulla ratio, and increased apoptotic cells in aged *Il-12b*$^{-/-}$mice compared with WT mice. There was, however, no apparent defect in thymic structure and thymocyte development in young *Il-12b*$^{-/-}$ mice. Surprisingly, in WT B6 mice, there was no age-related

decrease in the levels of IL-12 produced by thymic DCs. Stimulation of thymocytes with IL-12 alone also did not enhance the thymocyte proliferative response *in vitro*. IL-12, however, provided a strong synergistic effect to augment the IL-7 or IL-2 induced thymocyte proliferative response, especially in aged WT and *Il-12b*$^{-/-}$ mice. Our data strongly support the role of IL-12 as an enhancement cytokine, which acts through its interactions with other cytokines to maintain thymic T-cell function and development during ageing.

5 Gene Therapy for B-Cell Responses

For B-cells, the first consideration is maintaining the CD4 helper T-cell response. This involves chemokines including IL-4 and IL-10 to promote a Th2 response. IL-4 is an important B-cell cytokine to promote entry into the GC where the B-cells contact with CD4 T-cells and DCs, and undergo rapid proliferation, class switch recombination and somatic hypermutation. These B-cells then develop into a plasma blast and finally, a plasma cell that migrates to the BM as a long-lived high-affinity antibody secreting B-cell. Other mediators necessary for this process include CD40L produced by CD4 T-cells to interact with CD40 on B-cells, as well as cell surface costimulatory signals including CD80/CD86 that interact with CD28 on T-cells. More recently, other B-cell surface molecules including ICOS ligand and CTLA-4 have been found to be important [69, 70]. Of these molecules, IL-4 may be a target for maintenance of a B-cell response.

Although both the number and responsiveness of peripheral B-cells in aged mice remain relatively intact, there are dramatic changes in B-cell generation [71, 72]. Alterations in B-cell development include both a skewing of V-gene utilization, especially in cells responsive to phosphorylcholine, and a decrease in the generation of various developmental B-cell subsets [73, 74]. The altered representation of these subsets appears to be the consequence of two developmental blocks. The first developmental block occurs during the maturation of proB-cells and is evidenced by a decrease in the number of preB-cells. The second developmental block occurs at the earliest stage of sIg(+)-cell maturation (sIgM^{lo}). Because of this block in B-cell maturation, in spite of a decrease in incoming preB-cells, the number of sIgM^{lo} cells appears to increase in aged mice. Additionally, the time of residence of cells within this maturational stage increases dramatically, while the proportion of cells in more mature (sIgM^{hi}) stages of BM development are decreased. In addition to the decreased number of maturing BM B-cells, the population of splenic B-cells that represent recent BM emigrants (HAS ^{hi}) is markedly decreased. In the face of this decrease in newly emerging cells from the BM, the population of mature splenic B-cells is maintained by their increased longevity.

B lymphopoiesis in senescent mice is typically diminished and characterized by low preB-cell numbers. The transcription factors E2A, Pax-5, and STAT5 have been implicated in the differentiation, proliferation, and survival of B-cell precursors [75–77]. The impairment of B lymphopoiesis during old age is related at the molec-

ular level to the handling and turnover of these key transcriptional proteins. Alterations in the expression of E2A, Pax-5, and STAT5 may affect multiple stages of B-cell development, contribute to reduced B lymphopoiesis, and preface changes in the "read-out" of the BCR repertoire during immune senescence.

6 Gene Therapy to Improve the Age-Related Decline in T-cell Response

IL-2 family members, IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are important for T-cell survival. IL-2, IL-7 and IL-15 signal through a common gamma chain (Fig. 2). IL-2, IL-7 and IL-15, have promise for prevention of immune senescence and regeneration of T-cells. For maintenance of T-cells, IL-7 plays a key role to prevent thymic involution and to maintain the naïve T-cell repertoire. IL-7 also plays a key role in acting through the CD127 (IL-7R) to maintain the CD8 memory T-cell pool [78]. IL-15 is also important in the maintenance of memory CD8 T-cells, and together with IL-7, they appear to be important to a varying degree for homeostasis of memory CD4 cells [79]. Therefore, the IL-2, IL-7, and IL-15 family is the most likely candidate for T-cell gene therapy.

The ability of IL-2 to restore response to virus or tumor cells has been analyzed. Fayad et al. [80] utilized a papilloma virus (PV) pseudo-virus (PSVs) as a model for vaccine and gene delivery vector to investigate if increased IL-2 could enhance the immune response to vaccination. One-year-old mice orally immunized with PSV-LCMV exhibited a decreased sera IgA response and a decreased mucosal

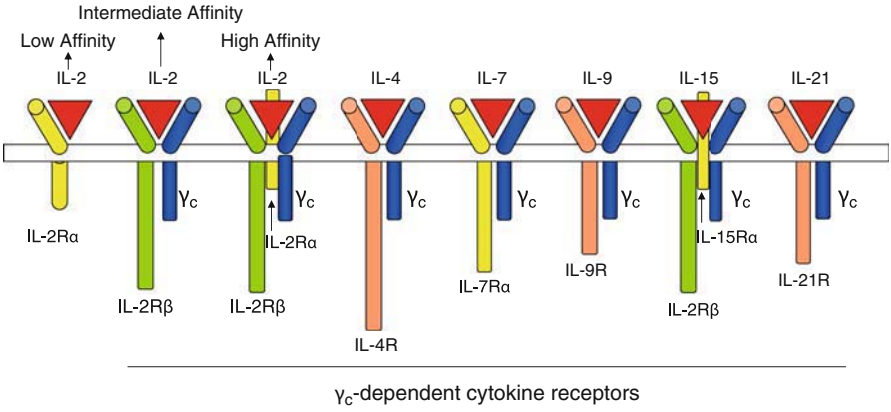

Fig. 2 IL-2R common gamma chain family. The γ_c cytokine family comprises interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21, named after the γ_c subunit (CD132) shared by receptor complexes for these cytokines. IL-4, IL-7, IL-9, and IL-21 bind a heterodimeric receptor comprised of the γ_c and the specific receptor subunits, IL-4Rα, IL-7Rα (CD127), IL-9Rα, and IL-21Rα chain, respectively. IL-2 and IL-15 bind a heterotrimeric receptor composed of the specific IL-2Rα (CD25) or IL-15Rα chain, and the shared IL-2/15Rβ (CD122) and γ_c chains

and systemic LCMV-specific CTL response. However, oral administration of a PSV expressing IL-2 augmented the generation of specific T-helper cells and protected aged mice, at least in mucosal viral challenge. This study demonstrated a novel approach to induce mucosal and systemic immune response in aged mice using PSV producing IL-2.

IL-2 could also enhance the age-related decline in response to tumor cells. Many studies have previously demonstrated that the injection of tumor cells genetically modified for the constitutive expression of cytokine into syngeneic immuno-complement mice resulted in enhanced activation of host-dependent antitumor responses [81–84].

The TS/A adenocarcinoma cell lines were engineered to express low, intermediate, or high levels of IL-2 [85]. TS/A cell lines expressing intermediate and high levels of IL-2 restored both the proliferative response of spleen T-cells in aged mice and the CTL response of aged mice. However, importantly, the TS/A IL-2 clones were not able to induce a tumor-specific immune memory response in aged mice suggesting that additional factors are necessary to confer an adequate tumor immune response.

7 IL-12 Gene Therapy to Improve the Age-Related Decrease in the CTL Response

Gene therapy for maintenance of the immune system has been thought to be potentially deleterious since normal immune senescence may be necessary to prevent increased states of inflammation at older age, or increased tendency for autoimmunity with age, or potentially increased development of lymphomas or leukemias. Therefore, as in other proliferative organs, the age-related decrease in cell cycle and proliferation may be difficult to safely reverse. An alternative strategy is to supply gene therapy for specific antigens and for limited periods of time to enhance the immune response *in vivo*. For example, for viral vaccination or for peptide antibody responses, in addition to better adjuvants, gene therapy may be necessary to temporarily replenish or rebuild the immune system prior to and after vaccination. IL-12 is an ideal candidate for such gene therapy.

Preclinical studies investigating new therapeutic principles against melanoma are presently being carried out in mouse models. Heinzerling et al. [86] have provided a different model using gray horses. These animals spontaneously develop metastatic melanoma that resembles human disease and is thus highly relevant for preclinical studies testing new immunotherapy protocols. Injection of plasmid DNA coding for the human cytokine IL-12 into established metastases induced significant regression in all 12 treated lesions in a total of seven horses. Complete disappearance was observed in one treated lesion, with no recurrence after 6 months. No adverse events have been observed in any of the animals during and after treatment. These results demonstrate the effectiveness and safety of IL-12 encoding plasmid DNA therapy against established metastatic disease in a large animal model and serve as a basis for a clinical trial.

We have carried out an AdIL-12 therapy to determine if this can correct age-related decline in CTL in aged mice. CD8 T-cells undergo rapid age-related senescence, with an attenuated capability to mount an effective CTL response in the ageing host, leading to a decreased ability to suppress viral growth and cancer [87]. Therefore, augmenting the CTL response is essential in ageing hosts. Our recent study showed that rapid successive administration of AdIL-12 vector and a subsequent injection of the wild-type Ad carrying the dominant antigenic peptide E1Bp can augment CTL activity in aged mice. Not only was the CTL activity increased, but the CTL activity was especially high at organ-specific sites to which the adenovirus exhibits high tropism. The CTL response in the sites of natural tropism for adenovirus, the liver and lung, was two to threefold increased relative to the CTL response in the spleen.

Aged mice exhibited higher serum levels of IL-12 and IFN-γ on days 3 and 7 after AdIL-12 administration compared to those of young mice. Also, a lower titer of anti-Ad antibodies in aged mice relative to that of young mice on day 3 after AdIL-12 administration may contribute to decreased viral clearance. However, there was no significant difference in the time of onset of detectable antibodies in aged mice compared to that of young mice which occurred on day 7 after AdIL-12 administration. We propose that the decline in both the innate response and adaptive immune response in the aged mice decreased viral clearance and therefore facilitated IL-12 production at specific target organs and augment CTL activity in these organs. In this way, our novel method of rapid successive administration made use of the decreased anti-viral immunity in aged mice to augment and expand the CTL response in these aged hosts.

Other investigators noted that IL-12 Rβ2 expression was deficient in $CD8^+$ CTL from old mice [88, 89] and the IL-12 ability to enhance T-cell functions is compromised with age as a consequence of changes in postreceptorial IL-12 signaling events involving signal transducer and activator of transcription 4 (STAT4) activation [90]. Our results suggest that increased IL-12 can overcome the defect. Furthermore, we observed an increase in antigen-driven proliferation of CD8 T-cells of virus-immunized aged mice after pretreated with AdIL-12. These results are consistent with previous findings that IL-12 promotes CD8 T-cell proliferation and such response was compromised in the absence/deficiency of IL-12 [91–93] indicating that *in vivo* delivery of IL-12 gene by an adenovirus vector can create a microenvironment that favorable to CTL response.

8 Chronic Inflammation as a Target for Gene Therapy

In addition to gene therapy for cytokines that are diminished with age, it is well known that certain proinflammatory cytokines have increased with age of deleterious effect. These cytokines include IL-1, IL-6, TNFα, and possibly IL-17. The gene therapy for inhibitors of IL-1 and TNFa has been extensively explored as treatments for arthritis. Such gene therapies include IL-1 receptor (IL-1Rα) expressed by both adenovirus and AAV, and soluble TNFR expressed by both adenovirus and retrovirus. In humans, Robbins and Evans have used retrovirus expressing IL-1Rα and TNFR for gene ther-

apy of arthritis in humans [25, 94]. Combined AAV gene therapy expressing sTNFR and IL-1RA has been used in mouse models of arthritis [95, 96].

9 Targeting Longevity Genes to Prevent Immune Senescence

Certain genes such as TERT can affect the rate of replicative cell senescence, including immune senescence. Such genes are candidate genes for prevention of immune senescence. Westin et al. [97] evaluated whether retroviral expression of TER and/or TERT, the catalytic component of telomerase, could extend telomere length and rescue autosomal dominant (AD) DC cells from a phenotype characteristic of early senescence caused by mutations in the telomerase RNA component (TER). Exogenous TER expression, without TERT, could not activate telomerase in AD DC skin fibroblasts. Transduction of TERT alone, however, provided AD DC cells with sufficient telomerase activity to extend average telomere length and proliferative capacity. Interestingly, we found that expression of TER and TERT together resulted in extension of lifespan and higher levels of telomerase and longer telomeres than expression of TERT alone in both AD DC and normal cells. These results provide evidence that AD DC cells can be rescued from defects in telomere maintenance and proliferation, and that coexpression of TERT and TER together provides a more efficient means to elongate telomeres than expression of TERT alone. Similar strategies may be useful for ameliorating the detrimental effects of telomere shortening in other diseases associated with telomerase or telomere defects [98]. Such proof-of-principle studies have led to screening for pharmacological approaches that might mimic the gene therapy effects, in a more clinically suitable formulation.

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Perspectives: Is Immunosenescence Clinically Relevant?

Tamas Fulop, Claudio Franceschi, Katsuiku Hirokawa and Graham Pawelec

Having read this book one can legitimately ask the question is immunosenescence clinically relevant and if it is so, what can be done for prevention, intervention and cure.

There is a large corpus of experimental data suggesting that the adaptive immune response, mainly the T-cell response, is deregulated with aging. Evidence is also accumulating to suggest that the innate immune response is altered as well. The exact causes of this deregulation are not known but for T-cells, thymic involution, changes in the distribution of subpopulations, and defective T-cell signal transduction are likely to be major contributors. The latter two alterations are likely to be the consequences of T-cell clonal exhaustion either by chronic antigenic stimulation or

T. Fulop (✉)
Tamas Fulop Research Center on Aging
Division of Geriatrics Dept of Medicine
Faculty of Medicine 1036 Rue Belvedere
Sherbrooke J1H 4C4 Canada
E-mail: tamas.fulop@usherbrooke.ca

C. Franceschi
Claudio Franceschi
CIG Interdepartmental Center "L.Galvani" University of Bologna
Department of Experimental Pathology
Via San Giacomo 12, 40126 Bologna Italy
E-mail: claudio.franceschi@unibo.it

K. Hirokawa
Katsuiku Hirokawa Institute for Health and Life Sciences
4-6-22 Kohinato Tokyo
Bunkyo-ku 112-0006 Japan

G. Pawelec
Universität Tübingen ZMF - Zentrum Med.Forschung
Abt. Transplant./ Immunologie Waldhörnlestr. 22
72072 Tübingen Germany
E-mail: graham.pavelec@uni-tuebingen.de; graham.pawelec@uni-tuebingen.de
Tel.: +49-7071-298-2805
Fax: +49-7071-294677

by metabolic alterations. It is of note that despite a wealth of data, the exact changes in the immune system with aging are still controversial because of the confounding influence of the physiological aging process and genetic as well as epigenetic factors, such as nutrition, neuroendocrine changes, chronic diseases, frailty. Thus, the field urgently needs to agree upon a set of biomarkers of immunosenescence to be applied preferably in careful longitudinal studies.

However, what we do know is that the changes in the immune response lead to various diseases and the book deliver a huge wealth of evidence for supporting this contention. The incidence of disease is dramatically increased with aging. These diseases are primarily of an infectious nature, but probably also include cancer, autoimmunity, and chronic inflammatory diseases. The definition and clinical use of an IRP to predict those at risk of incipient mortality could facilitate a major breakthrough in the prevention and modulation of immune-related morbidity and mortality.

Knowing the importance of the immunosenescence in diseases, some means to intervene in compensating for immune deregulation which could be applied in the elderly without ethical or regulatory problems do already exist. These include balanced nutrition in macro and micronutrients, including functional foods such as vegetables and fruit, functional foods such as probiotic-containing yoghurt, sustained moderate aerobic exercise regimens, and could be relatively easily extended to include better vaccines and vaccination strategies against different pathogens especially against influenza, pneumococcus pneumoniae, herpes zoster and in the near future against CMV, and possibly application of certain antiviral drugs and low-dose cytokines.

Nevertheless, there is still a long way to go to implement more specific and effective safe immunorestorative therapies, as suggested in this book, even at the level of specific nutrients or drugs. Thus, a better understanding of immunosenescence by intensive basic and clinical research and the development of new methods and strategies to intervene in its evolution are essential for improving the quality of life of the increasingly large elderly population.

We thank all the authors who contributed to this book for finally answering to this burning question and indicating hope and directions for the future of the immunosenescence.

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