Edited by Manfred B. Lutz, N. Romani and A. Steinkasserer

Handbook of Dendritic Cells

Biology, Diseases and Therapies

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Volume 1

Edited by
Manfred B. Lutz, Nikolaus Romani,
and Alexander Steinkasserer

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With an Introduction by
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Colored scanning electron microscope pictures of dendritic cells. (Courtesy of Prof. Dr. Kristian Pfaller, Innsbruck University, Austria.)

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Preface

Time flies.... Life, including research, is becoming ever faster. Papers are published online long before they appear in print. So, why such an "old-fashioned" book? Ralph Steinman and Jacques Banchereau gave a good answer to this concern in the "predecessor book" to this volume: the first edition of the Dendritic Cell book edited by Michael Lotze and Angus Thomson in 1999 [1]. Books may serve as a comprehensive historical record of the state-of-the-art at a given time. Even books published as long ago as 1991, such as Gerold Schuler's volume on Langerhans Cells [2], still provide a valuable source of knowledge, not only for novices to the field. For these reasons we felt that our attempt to assemble yet another such book was justified and would provide a useful service to the scientific community.

Five years have elapsed since the second edition of above-mentioned dendritic cell book. Dendritic cell research has proceeded tremendously during this period, and has increasingly been linked with clinical research. Clearly, the time is ripe to have another such reference volume on our shelves and desks to browse through, search, find and sometimes perhaps even remember. We have encouraged the contributors to also look back in time and to try and put recent data into a wider perspective.

Most of the relevant issues in dendritic cell biology have been covered in this book. Of course, nothing is perfect, and some interesting and important areas have not been discussed. To obtain a critical synopsis of the scope of this volume we recommend the reader to enjoy Ralph Steinman's introductory chapter as an "apéritif".

We thank all contributors to this book for their great efforts and for their time. All of us have multiple commitments, and "besides this", many of us have children who'd often like to do something else than just watch their mothers or fathers writing papers..... Therefore, we appreciate these efforts even more, and we express our thanks to all those who encouraged us to undertake this endeavour. We also thank our editorial partner, Dr. Andreas Sendtko from Wiley-VCH and Brigitte Wölfel from Erlangen, who managed the transformation of a pile of individual manuscripts into a nice book smoothly and efficiently.

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Finally, we are indebted to our teachers and mentors, above all Ralph Steinman and Gerold Schuler, who made it possible that we are now in the position to edit such a book.

Enjoy the book, and enjoy dendritic cell research!

Manfred B. Lutz
Niki Romani
and Alexander Steinkasserer

Erlangen and Innsbruck, January 2006

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Part A
Dendritic Cell Biology

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1
Introduction to Some of the Issues and Mysteries Considered in this Book on Dendritic Cells

Ralph M. Steinman

The authors, under the leadership of Manfred Lutz, Nikolaus Romani and Alexander Steinkasserer, are to be congratulated for assembling this timely volume on dendritic cells. This book will help all of us to keep up! The special position of dendritic cells in the immune system can be summarized in some general terms. Lymphocytes have exquisite mechanisms for recognizing an infinite array of self and foreign antigens, but they require dendritic cells for many critical functions. Dendritic cells use a specialized endocytic system to capture antigens for processing and display to lymphocytes. Dendritic cells respond to a plethora of stimuli, not only pathogen components but also endogenous ligands including cytokines. While presenting self and foreign antigens, dendritic cells exhibit migratory, homing and lymphocyte binding properties that allow clonal selection to take place. Following clonal selection, dendritic cells influence a key decision, whether lymphocytes are to be tolerized or immunized, and for the latter, dendritic cells control the quality of the immune response through the regulated expression of many co-stimulatory molecules. The purpose of this introduction is to briefly summarize what lies ahead in the chapters of the book, and to do this by restating some of the issues and mysteries that they will consider.

1.1
Dendritic Cells as a Distinct Hematopoietic Lineage

1.1.1
Chapters 1–16, the Life History of Dendritic Cells

The cytokine flt-3L is currently the most effective and selective way to expand the output of many types of dendritic cells, and dendritic cell progenitors can be enriched by their expression of flt-3 [1]. The dendritic cells enter the blood and tissues in precursor and immature forms where they act as sentinels, poised to capture antigens and to respond to an array of environmental cues. An important position for dendritic cells is at mucosal surfaces, where self antigens [2] and microbial

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products [3–5] are captured. The information obtained by dendritic cells – both antigens and other stimuli for dendritic cell maturation – is then conveyed to T cells and other kinds of lymphocytes, primarily in lymphoid tissues where dendritic cells encounter innate (NK, NKT) and adaptive (B, T) lymphocytes. At this point, the life span of dendritic cells is limited [6], and the cells do not leave the lymphoid organ because they are not found in efferent lymph [7]. However, there is no doubt that dendritic cells can additionally accumulate in peripheral tissues, for example at sites of delayed type hypersensitivity, and even help to organize lymphoid tissue-like structures in chronic inflammatory disease [8].

1.1.2 Questions Concerning the Dendritic Cell Lineage

As one reads chapters 1–16, many current unknowns will surface. Are there any functional differences between dendritic cells when they arise from lymphoid as opposed to myeloid progenitors? Are the immature dendritic cell progeny that are expanded by flt-3L only committed to become dendritic cells, or can they still "transdifferentiate" to become other types of phagocytes or lymphocytes? What transcription factors control dendritic cell development including distinct subsets? What is the origin of dendritic cells in lymphoid tissues in the steady state? Can some dendritic cells in lymphoid tissues originate directly from the myeloid and plasmacytoid dendritic cell populations in the blood, or do most dendritic cells (also monocytes and subsets of monocytes) first patrol peripheral tissues before moving to the lymphoid organs? What factors are responsible for the entry of dendritic cells from tissues into the afferent lymph in the steady state? This flux is postulated to allow dendritic cells to bring samples of self tissues and environmental proteins to the lymph nodes for purposes of tolerance. What is the raison d'être for subsets of dendritic cells? Are they programmed to carry out distinct innate responses by expressing distinct receptors for antigen uptake, toll ligands, and cytokines? If antigens are successfully processed, are all dendritic cell subsets capable of mediating similar forms of tolerance and immunity, depending upon their maturation state? These topics are pertinent to the understanding of the dendritic cell lineage and the control of many aspects of immune function.

1.2 Control of Lymphocyte Responses by Dendritic Cells

1.2.1 Chapters 17–30, Initiation of Immunity

The classical emphasis in dendritic cell biology has been to understand the initiation of T-cell immunity. This remains a focus of chapters 17–30, but attention is also given to more recently appreciated roles of dendritic cells in stimulating other types of lymphocytes and controlling antigen-specific tolerance. There are several

sets of requisite features that dendritic cells express. These include (i) specialized receptors for antigen uptake and efficient processing pathways, including the mysterious cross presentation pathway, whereby non-replicating antigens are processed for presentation on MHC class I and now on other molecules like CD1 [9, 10]; (ii) the production of many membrane co-stimulators (from the B7, TNF and Notch families) as well as cytokines and chemokines; (iii) a group of migratory, homing and lymphocyte binding functions that allow dendritic cells to survey the periphery and move to lymphoid tissues; and (iv) the presence of distinct subsets that can carry out different forms of innate and adaptive resistance. I would like to consider some issues with regard to the first two topics, which are considered at many points in this volume.

1.2.2 Questions Concerning Antigen Uptake, Processing and Presentation

Although dendritic cells have many potential receptors for adsorptive endocytosis, what are the natural ligands for many of these such as DEC-205/CD205, langerin/CD207, and a host of other lectins? Interestingly, antibodies to these receptors can be engineered to express defined antigens, and this would seem to be an important new way to analyze receptor and dendritic cell function *in vivo* [11, 12]. Do these receptors function exclusively to enhance antigen capture, or are they additionally specialized to navigate special processing pathways within the cell and/or to couple with other signaling receptors such as toll like receptors? Might the presentation of “exogenous” antigens on MHC class I best be figured out in dendritic cells, which are so efficient at this pathway *in vivo* following capture of dying cells, immune complexes, and ligands for DEC-205? What underlies the distinct regulation of antigen presentation in dendritic cells, which seems different in different sites? For example, dendritic cells that are derived from bone marrow precursors in culture, as well as Langerhans cells, can markedly increase the efficiency of antigen processing and MHC peptide complex formation during dendritic cell maturation [13–15]. Nonetheless, some steady state dendritic cells in peripheral lymphoid organs are continuously able to form at least some MHC peptide complexes for purposes of immune tolerance [11, 12]. What is the potential role of dendritic cells in direct antigen presentation to B cells, where native antigens are to be recognized [16]? How are dendritic cells recognized by NK lymphocytes [17–20]?

1.2.3 Questions Concerning Dendritic Cell Maturation

Maturation has been an important concept, first historically, because it stated that dendritic cells not only had to capture antigens but also had to differentiate extensively to initiate immunity [21, 22]. At the time, the terms “accessory” and “sensitizing” functions rather than co-stimulation were in use to describe the special roles of dendritic cells beyond antigen processing. Second maturation is really the critical link between innate and many forms of adaptive immunity wherein lym-

phocytes differentiate along many different lines and with important consequences: distinct types of effector cells, long term clonal expansion, and memory. The single term "maturation" clearly cannot specify the many different responses that dendritic cells exhibit when they encounter endogenous (CD40L, thymic stromal lymphopoietin and other cytokines) and exogenous (ligands for Toll-like receptors) stimuli. Nevertheless, I regard "maturation" to be a much better word than "activation" because an intricate and often irreversible process of differentiation takes place [23] rather than a relatively restricted on-off response.

Some of current questions in this central field will be apparent on reading chapters 17–30. First, does the same dendritic cell determine the quality of a lymphocyte response, e.g. tolerance vs. immunity, CD4 vs. CD8 responses, Th1 vs. Th2, or are there distinct dendritic cells that are devoted to the control of these different key outcomes of antigen presentation? Second, how must dendritic cells differentiate to become potent stimulators of Th1 type CD4 responses and CD8 killer responses? It has been believed for some time that this required signal one (MHC peptide) and signal two (B7 co-stimulators), but recent evidence shows that additional differentiation mediated via CD40 is required, even after dendritic cells are successfully presenting MHC peptide complexes and expressing high levels of co-stimulatory molecules, both membrane bound and cytokines [24]. These observations were made with NKT lymphocytes as inducers of dendritic cell maturation, but the same may well be true for microbial stimuli. Third, what types of dendritic cell products are needed for different types of responses? With respect to the key Th1 vs. Th2 decision, new players other than IL-12 need to be considered, e.g. the recent report that dendritic cells use delta and jagged Notch ligands to elicit Th1 and Th2 responses respectively [25]. A long neglected topic is the importance of dendritic cells in memory. Dendritic cells can induce memory, but how? Fifth, how do dendritic cells select the type of lymphocyte that they will interact with? Are all maturing dendritic cells able to interact with NK, NKT, T and B cells, or does the maturation stimulus and dendritic cell subset govern the outcome? This a long list of unknowns, but they pertain to issues of broad impact in immunology.

1.3 Dendritic Cells in Disease Pathogenesis

1.3.1 Chapters 31–51, Dendritic Cells in Infectious and Other Diseases

The interface of microbiology with immunology has been energized by the discovery that Toll-like receptors mediate recognition of a diverse array of microbial ligands [26, 27], and as one consequence, drive the maturation of dendritic cells [28–30]. Many other central areas of medicine also involve hematopoietic cells and immune responses – transplantation, allergy, autoimmunity, cancer, even it now appears, neurodegeneration and atherosclerosis. Immunology can contribute significantly to these prevalent and enigmatic diseases. Chapters 31–51 provide exam-

ples in which dendritic cells are being investigated to understand the development of disease.

1.3.2 Some Questions on the Roles of Dendritic Cells in Diseases

In transplantation, we need to understand more about the initiation of immunity, i.e. what processes in the graft allow dendritic cells to initiate immunity both by the direct pathway (graft dendritic cells present antigens to host T cells) and indirect pathway (host dendritic cells present antigens from the graft)? In allergy, there are real mysteries on how dendritic cells polarize to Th2, particularly in the lung, where this seems to be a Th2 prone environment. There is exciting data that thymic stromal lymphopoietin made by epithelial cells condition a subset of myeloid dendritic cells to induce "inflammatory Th2 cells" (T cells that make not only IL-4 but also TNFα instead of IL-10) [31]. In autoimmunity, particularly lupus, blood monocytes are differentiating along a dendritic cell pathway [32], and this may lead to immunity to self antigens, particularly complexes of autoantigens and antibody [33, 34]. Dendritic cells may be part of a circuit that allows a basic defect in autoantibody formation [35] to bring about autoimmunity. Immune complexes can trigger conventional [33] and plasmacytoid dendritic cells [34] to produce type I interferon, and these interferons may also help expand autoreactive T cells, which in turn increase the switching and affinity of autoreactive B cells. Likewise, the hygiene hypothesis may transpire via dendritic cells. Exposure to microbial stimuli leads to dendritic cell maturation, and the maturing cells are able to induce different types of regulatory and suppressor cells that suppress allergy and autoimmunity [36–38]. Can the immune system be energized against cancer by dampening the mechanisms used by tumors to suppress dendritic cell function, especially maturation [39]? And as the chapters will discuss, many examples of chronic infection are now being analyzed at the levels of dendritic cells. HIV remains the most urgent, since the virus may co-opt dendritic cells to enhance replication in T cells, to induce regulatory cells, and to block maturation [40]. This area of research is limited by the many demands of doing human studies [41]. It will be important for the scientific community to have the conviction to overcome these obstacles, since significant and challenging scientific issues need to be addressed.

1.4 Dendritic Cells and the Design of Vaccines and New Therapies

1.4.1 Chapters 52–55, Dendritic Cells in Immunotherapy

It is not simply a matter of "applied science" to identify new preventions and therapies. Rather the identification of new cures and treatments provide challenging questions for research. The final chapters consider some of these issues. How can

tumor antigens be delivered to dendritic cells, and what maturation stimuli are best to use, especially ex vivo where dendritic cells are potentially exciting adjuvants for active immunization [42, 43]? Can dendritic cells be mobilized to bring about therapeutic immunity? Does the newly recognized capacity of dendritic cells to induce suppressor T cells interfere with immune therapy, but on the other hand, provide new ways to treat autoimmunity and allergy and transplant rejection? Again, the stage is set for immunology to contribute to the design of new preventions and therapies, and dendritic cell biology will be an important part of these initiatives.

1.4.2
Dendritic Cells and the Design of Vaccines against Infectious Diseases

One area that is not yet well developed, and therefore not addressed extensively by this book, is that of the immunological approach to vaccines against global infectious diseases. The international effort remains exclusively directed to microbial approaches and microbial vectors. It is perplexing that immunology has not been able to contribute more to the urgent need for new vaccines, but I think this will change. With the need for more effective vaccines, particularly in infections like HIV, malaria and tuberculosis – where cell mediated immunity is likely to be critical – segments of the scientific community are coming to understand that one cannot simply ordain that this or that strategy is “immunogenic” without trying to understand the basis for vaccine efficacy or inefficacy and the correlates of protection. Nevertheless, the field still views vaccines in a bipartite manner, vaccines plus lymphocytes, whereas there is a critical third party or intermediary, dendritic cells.

Many obstacles, imposed by the pathogen, need to be considered in developing vaccines against HIV, TB, malaria and others. However, I believe that the central current obstacle is the need for research to learn to elicit strong immunity and memory in patients, especially T-cell mediated immunity. The many adjuvant roles of dendritic cells, if harnessed, could prove fruitful [44]. It is hoped that some of the increasing resources that are now being directed to vaccine design will bring this about. This book portrays a ménage a trois – antigens, lymphocytes and dendritic cells – and provides valuable perspectives to overcome the gap in identifying antigen specific vaccines and therapies.

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I
Dendritic Cell Development

2
Bone Marrow Progenitors of Dendritic and Natural Interferon-producing Cells

Markus G. Manz

Dendritic cells (DCs) as well as natural, type I interferon-producing cells (IPCs, also called plasmacytoid dendritic cells, pDCs) are part of the hematopoietic system [1]. In mice, and probably similarly in humans, DCs and IPCs have a short *in vivo* steady-state turnover time of about four and fourteen days respectively, as determined by BrdU labeling experiments [2, 3]. Since tissue DCs and IPCs are nondividing cells, they need to be regenerated continuously via developmental intermediates from hematopoietic stem cells (HSCs). This process must be tightly regulated. What are the critical factors that determine DC and IPC development *in vivo*; which successive transcriptional events must occur; what are the essential cytokines and chemokines; along which major hematopoietic pathways do developmental intermediates segregate; and is there a regulatory feedback loop that signals fluctuating need for DC and IPC regeneration in steady-state and inflammation? With improving technologies to purify minute viable cellular fractions by fluorescence associated cell sorting (FACS), to track transferred cells *in vivo*, to flag-tag, delete, or over-express candidate critical genes in experimental animals, and with the availability of recombinant cytokines, tremendous progress has been made over the last few years regarding the answers to these questions. Most of these findings are summarized in excellent recent reviews (e.g. [4–8]) and many contributing scientists are authors of the following chapters. Thus, I will focus here mainly on our and others' findings on DC and IPC development from early hematopoietic progenitor cells isolated from mouse bone marrow or human cord blood, and, based on this data, will propose a revised working model for early hematopoietic steady-state DC and IPC development.

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2.1 Hematopoietic Stem Cells and Successive Lineage-restricted Early Hematopoietic Progenitor Cells

The differentiation of stem cells to mature cells is regarded as a unidirectional process, characterized by successive loss of self-renewal capacity and developmental options, and by final restriction to a terminally differentiated mature cell type [9].

Currently, hematopoiesis is one of the mammal adult stem cell differentiation systems that has been most studied. In both mice and men, HSCs as well as successive multiple developmental intermediates at different branch points, with limited cellular expansion potential and restriction to specific mature cell types, have been isolated from bone marrow and cord blood to high purity according to their specific surface phenotype by FACS (for review, see [1]). Consecutively, a phenotypic and functionally-defined hematopoietic developmental map has been drawn where long-term HSCs (LT-HSCs) with life-long self-renewal capacity can give rise to short-term HSCs (ST-HSCs) with limited self-renewal capacity that then give rise to non-self-renewing, multipotent progenitors (MPP) (e.g. [10, 11]). With the isolation of mouse clonal common lymphoid progenitors (CLPs) [12] and clonal common myeloid progenitors (CMPs) [13] and their respective human counterparts [14–16] the long-postulated hypothesis for an early lympho–myeloid dichotomy as an alternative and exclusive differentiation step for MPPs is strongly supported. CLPs are clonal progenitors for both B and T cells *in vitro* and mouse CLPs possess rapid and potent T, B, and NK cell, but no myeloid differentiation activity. Just 1000 CLP can generate $0.6–1.1\times10^7$ $CD3^+$ spleen T cells in 4–6 weeks, and 1.4×10^7 $B220^+$ spleen B cells in 2 weeks *in vivo* [12], and CLPs co-transplanted in addition to HSCs in autologous or allogeneic transplantation models, can rescue mice from otherwise lethal mCMV infections [17]. CMPs are clonal progenitors for both megakaryocytes/erythrocytes and granulocytes/macrophages *in vitro*, and as a population they give rise to megakaryocyte/erythrocyte (MEPs) and granulocyte/macrophage progenitor cells (GMPs) [13, 16]. While mouse CMPs contain few (about 1:2700) cells with B-cell developmental potential, T-cell development is never observed, and offspring MEPs and GMPs harbor no lymphoid potential [13]. Upon *in vivo* transfer, CMPs generate all myeloid cells, MEPs exclusively generate megakaryocytes and erythrocytes, and GMPs exclusively generate granulocytes and macrophages with high efficacy: CMPs and MEPs are capable of conferring radioprotection in lethally irradiated animals [18], and CMPs and GMPs protect against invasive aspergillosis and *Pseudomonas aeruginosa* infection following hematopoietic stem cell transplantation or chemotherapy in experimental mice [19, 20].

Based on these findings, mouse CLPs, CMPs, MEPs, and GMPs are currently the most extensively phenotypically- and functionally-defined early restricted bone marrow progenitor cells. Given their frequency, their cycling status, and their *in vivo* reconstitution potential, CLPs and CMPs could theoretically be developmental intermediates for most, if not all, mature lymphoid and myeloid cells, respectively [1]. However, it is so far not formally proven whether they are indispensable, or

whether alternative developmental pathways might exist. In fact, the concept that CLPs are the major physiologic T-cell progenitors is questioned by experiments where HSCs, CLPs, and thymus-derived progenitors (early thymocyte progenitors, ETPs) were directly compared: ETPs were capable of generating more and longer T-cell progeny than CLPs *in vivo*, and, in contrast to CLPs, had some myeloid potential [21]. Thus, possibly ETPs arise from circulating, thymus-homing HSCs, MPPs, or alternative early progenitors without proceeding through a CLP state [22–25]. Although these findings do not challenge the physiologic importance of common lymphoid or myeloid restricted progenitors, they point to an important issue that might not be addressed appropriately by current *in vivo* cellular transfer models: during successive lineage commitment, progenitors are generated that incline to a lineage but might use alternative developmental options upon changing environmental stimuli. Thus, directed or random migration of progenitor cells as well as experimentally induced changes of compartments could greatly impact on progenitor contribution to any given mature cell lineage.

2.2
Proposed Models for Dendritic and Natural Interferon-producing Cell Differentiation

DCs were originally described by their capacity to efficiently process and present antigens, and to prime naïve T cells [26]. Over the last three decades, multiple DC subtypes have been defined, differing in phenotype, localization and immune function [6, 27]. With the recent phenotypic and functional identification of IPCs in both men [28–30] and mice [31–33], and the finding that these cells are capable of differentiating to DCs, the heterogeneous group of DCs has been further enlarged [8] (details on different DCs are discussed in the respective chapters of this book). With the exception of Langerhans cells (LCs) [34], DCs and IPCs have a short steady-state turnover time of about four and fourteen days in lymphoid tissues, respectively [2, 3]. Thus, they need to be continuously renewed from HSCs via intermediate progenitors.

How are steady-state DCs and IPCs generated from HSCs and which developmental intermediates are involved? Two principal and opposing models have been proposed: a) a “specialized lineage” or “evolutionary selection” model, where different DC subtypes belong to different hematopoietic lineages that are determined at the level of early hematopoietic progenitors; b) a “functional plasticity” or “environmental instruction” model, where different DC subtypes belong to one hematopoietic lineage and are determined at the level of an immediate DC precursor, depending on local influences.

The concept of specialized DC lineages was strongly supported along two lines of evidence in mice and men: Firstly, due to the exciting discovery that mouse thymocyte progenitors *in vivo* are capable of producing CD8α expressing DCs, a population that accounts for most DCs in mouse thymus and about one third of DCs in secondary lymphoid organs (see also Chapter 12), a T-cell development associated CD8α lymphoid DC lineage was suggested [35, 36]. This concept seemed to be

further supported by the findings that thymocyte progenitors give rise to DCs *in vitro* without the need of myeloid development-associated granulocyte-macrophage colony-stimulating factor (GM-CSF) [37], and that mice deficient in transcription factors Ikaros [38], RelB [39], and PU.1 [40] lack only CD8α negative DCs (see also Chapter 4). Secondly, it was suggested that IPCs in humans (and later also in mice) are of lymphoid lineage origin based on the following indirect findings: human IPCs express CD2, CD4, CD5, and CD7 but not CD11c, CD13, CD33 and mannose receptors [8, 28]; IPCs express T-cell development-associated pre-Tα [41–43], and B-cell development-associated mRNA transcripts Igλ-like 14.1 and Spi-B [43, 44]; GM-CSF does not promote IPC development [45, 46]; and *in vitro* differentiation of IPCs and B cells is inhibited by ectopic expression of inhibitor of DNA binding Id2 and Id3 [42].

While the above findings provide important information regarding the biology of DCs and IPCs, the interpretation in terms of developmental association and shared differentiation with defined hematopoietic lineages remained inconclusive for several reasons: a) the assumption that all CD8α DCs were lymphoid-derived because thymocyte progenitors-generated CD8α DCs could at that time not be controlled by respective "opposing" myeloid restricted progenitors; b) the presence or lack of certain DC subtypes in combination with other hematopoietic cells in genetically-targeted mice or human cells does not necessarily imply that the gene of interest is involved at a common developmental checkpoint for both cell types; rather, certain genes might be used independently in different pathways (this was actually suggested by other gene-target experiments discussed in later chapters); c) the presence or lack of mRNA transcripts or intracellular and surface proteins in DCs and IPCs, that were initially defined in a cell type other than in DCs or IPCs, does not necessarily imply that both cells are derived from a common immediate precursor; d) finally, the expression of cytokine receptors and the response to the cognate cytokine does not ascribe all expressing/responding cells to a defined common hematopoietic lineage.

Thus, given these restrictions, the most direct way to identify hematopoietic lineage relationships is to isolate intermediate precursors to purity, to test their transcriptional profile, and to evaluate their full *in vitro* and *in vivo* developmental potential, both as a population and on a clonal level.

2.3 Unexpected Redundancy in Dendritic and Natural Interferon-producing Cell Development from both Lymphoid and Myeloid Restricted Hematopoietic Progenitor Cells

With the isolation of mouse CLPs [12], pro-T cells [47], pro-B cells [48], CMPs, GMPs, and MEPs [13], and the isolation of their respective human counterparts [14–16], it became possible to test directly the DC and IPC developmental capacities from each lineage restricted progenitor population.

By doing so in both *in vitro* and *in vivo* assays, we and other laboratories came to several surprising findings, first in mice and then in men: 1) mouse CLPs as well

as CMPs generate functional CD8α positive and CD8α negative DCs *in vivo* [49–51]; 2) differentiation activity into both CD8α positive and CD8α negative DC is preserved in early T-cell progenitors (pro-T1), declining along T-cell maturation (pro-T2), as well as in GMPs [50, 51]; 3) at least in our hands, *in vivo* transfer of progenitors in lethally irradiated animals preferentially leads to CD8α positive DCs development irrespective of the progenitor transplanted [49, 50]; 4) as in DC development, CLPs, pro-T cells, CMPs, and GMPs generated functional IPCs *in vivo* [52–54]; 5) if most DCs and IPCs develop through either a CLP or CMP state, and if DC and IPC reconstitution capacities reflect *in vivo* steady-state DC and IPC development, we calculate that most of secondary tissue DCs and IPCs are of CMP origin and that both CMP and CLP reconstitute about half of thymus DCs and IPCs, respectively [49, 50, 52, 54]; 6) DC and IPC developmental potential in myeloid progenitors is not due to the minor B cell developmental capacity of CMPs because on a per cell basis, DC and IPC developmental capacity is higher than B-cell capacity in CMPs, and because GMPs develop to DCs and IPCs but not to B cells [50, 52, 54]; 7) both DC and IPC developmental capacities are lost, once definitive B-cell commitment or megakaryocyte/erythrocyte commitment occurs [50, 52] (we were not able to repeat reported rare *in vitro* DC development from pro-B cells [55]); 8) finally, as in mice, human CMPs and GMPs, as well as lymphoid progenitors but not MEPs, are capable of generating DCs and IPCs *in vitro* with clonal DC/IPC capacity being about fivefold higher in myeloid compared to lymphoid progenitors [56].

Thus, taken together, these experiments revealed, in both mouse and human hematopoietic development, a so far unique and unexpected redundancy in DC and IPC development from both common lymphoid and T-cell committed (CLPs, pro-T cells), as well as common myeloid and granulocyte/macrophage-committed (CMPs, GMPs), hematopoietic progenitor cell populations that is, however, terminated once definitive B cell or megakaryocyte/erythrocyte commitment occurs.

2.4 Immediate Dendritic and Natural Interferon-producing Cell Precursors

The data discussed above demonstrate redundant DC and IPC development from different hematopoietic lineages. However, it does not define restricted clonal DC or IPC, or restricted clonal common DC/IPC progenitors, i.e. progenitor cells capable of producing all types of DCs, IPCs, or both DCs and IPCs on a single cell level without giving rise to any other cell type. Both DCs and IPCs are relatively rare cells. Upon *in vivo* transplantation, mouse early lymphoid and myeloid progenitors can produce more than 10 000-fold progeny lymphoid or myeloid cells [13, 57], however, they only produce about tenfold progeny DC and IPC compared to input numbers [50–52]. Similarly, when tested as a population or on a single cell basis *in vitro*, lymphoid and myeloid progenitors divide only little (3–8 times) to become nondividing DCs or IPCs [50, 56]. If early lymphoid or myeloid progenitors are upstream of a restricted clonal common DC, IPC, or DC/IPC precursor, this

precursor will probably have low proliferative capacity. Thus defining it will be technically demanding.

Recently, potential candidates have been identified in mice: first, a CD11c positive MHC class II negative cell fraction in blood was reported to give rise to all DC populations as well as to IPCs *in vivo*, while other cell types were not produced, at least at early time points analyzed after transplantation [58]; second, a Ly6C, CD31, and CD11c positive population, termed "pre-immunocyte" in bone marrow generates monocytes, CD8a positive and negative DCs, as well as IPCs without significant cell division [59]. However, clonal experiments were not reported and thus it will now be important to further determine the homo- or heterogeneity of the two populations.

2.5
Proposed "flt3-license" Working Model for Steady-State Dendritic and Natural Interferon-producing Cell Development from Early Hematopoietic Progenitor Cells

If both DCs and IPCs develop along otherwise lineage-restricted hematopoietic pathways, what then is the "unifying element" that might define readiness of progenitors to receive and execute signals that drive DC and IPC development?

To better understand this, we and others took a closer look at flt3 receptor/flt3-ligand, a nonredundant cytokine receptor/ligand pair for steady-state DC and IPC development: while granulocyte/macrophage colony-stimulating factor (GM-CSF) deficient or M-CSF deficient (op/op) mice show no substantial changes in their lymphoid organ DC and IPC compartments [60, 61], flt3-ligand deficient mice and mice with hematopoietic system confined deletions of Stat3, a transcription factor activated in the flt3 signaling cascade, show massively reduced DCs and IPCs [62, 63] (and R. Tussiwand and M.G. Manz, unpublished data); injection of flt3-ligand or conditional expression of flt3-ligand in mice increases DC and IPCs, with up to 30% of mouse spleen cells expressing CD11c [32, 64–69], and similar as in mice, flt3-ligand injection increases both blood levels of DCs and IPCs in humans [70, 71]; furthermore, flt3-ligand as a single cytokine is capable of inducing parallel *in vitro* differentiation of both DCs and IPCs from mouse bone marrow cells as well as from human $CD34^+$ cells [45, 56, 72]. Flt3 is a receptor tyrosine kinase with homology to c-Kit (the receptor for stem cell factor, SCF) and c-fms (the receptor for macrophage colony-stimulating factor, M-CSF) (reviewed in [73]). Flt3 receptor was shown to be expressed on short-term (ST-) reconstituting, but not on long-term (LT-) reconstituting, HSCs in mice [10, 11]. We further mapped expression of flt3 mRNA and surface protein along the hematopoietic tree: flt3 is transiently upregulated from ST-HSCs on MPPs on most of CLPs (60–70%) and CMPs (50–65%); flt3 is maintained at low levels in earliest pro-B cells (pro-B(A)) [48], pro-T cells, and GMPs, while it is downregulated and lost in definitive B cell (pro-B(B/C)), T cell, and megakaryocyte/erythrocyte (MEP) lineage commitment [67]; flt3 is further expressed on all steady-state, *ex vivo* isolated lymphoid tissue DCs and IPCs but not on other steady-state hematopoietic cell lineages, and, upon flt3-

ligand application, $flt3^+$, but no or few $flt3^-$ progenitors, as well as DCs and IPCs, are expanded [67]; furthermore, both CLP and CMP offspring DCs increase in numbers upon flt3-ligand application, thus proving that flt3-ligand drives DC development along both lymphoid and myeloid development associated pathways [67]. In contrast to $flt3^+$ progenitors, $flt3^-$ progenitors (as e.g. MEPs), did not give rise to DCs and IPCs *in vitro* and *in vivo*, and thus DC and IPC development is confined to flt3 expressing hematopoietic progenitor cells [53, 67].

If, as suggested by these findings, flt3 is capable of delivering an instructive signal for DC and IPC development, enforced expression of flt3 in flt3 negative progenitors might re-activate or rescue DC and IPC developmental options. Indeed, expression of human flt3 in mouse $flt3^-$-progenitors rescues IPC/DC-differentiation capacities to levels of $flt3^+$-progenitors. This is accompanied by hu-flt3-induced upregulation of DC- and GM-development affiliated genes. Furthermore, huFlt3-expression in $flt3^+$ progenitors enhances IPC/DC development (N. Onai and M.G. Manz, unpublished data). Thus, these findings prove that enforced flt3 signaling is sufficient to instruct IPC/DC-, and GM-differentiation programs in $flt3^-$ progenitors, and suggests that flt3 signal strength might be the earliest regulator of DC/IPC development.

Based on this, it is reasonable to propose a "flt3-license" working model for steady-state dendritic and natural interferon-producing cell development from early hematopoietic progenitors (Fig. 2.1). In this both DC and IPC developmental capacities are maintained in proliferating, flt3-expressing cells, irrespective of their loss of alternative (i.e. lymphoid or myeloid) developmental options. This would be the steady-state "flt3-license pathway" for DC and IPC development (Fig. 2.1, pathway A). If flt3 is downregulated, DC and IPC developmental capacities are lost in proliferating cells (Fig. 2.1, pathway B), e.g. in megakaryocyte/erythrocyte progenitors. Alternatively, flt3 expressing, proliferating cells might lose DC and IPC developmental capacities by competitively activated, dominant lineage-restriction signals (Fig. 2.1, pathway C), e.g. by the upregulation of Pax5 transcription factor in B-cell development [74].

While this "flt3-license" working model is suitable for steady-state DC and IPC development, how can DC development of from nonproliferating $flt3^-$ monocytes [67] be integrated? Although DCs generated from circulating human monocytes in GM-CSF and IL-4 supplemented cultures are a major source for human DCs in preclinical and clinical studies [75, 76], GM-CSF was not shown to be an essential cytokine for mouse steady-state DC development, as mentioned above [60]. In fact, *in vivo* DC development from monocytes was not observed in steady-state but only upon inflammatory stimuli, e.g. when monocytes were transferred in irradiated mice (but not upon transfer in nonirradiated mice) [77], in mice with inflamed peritoneal cavities [78], or when local inflammatory stimuli were applied to skin [79]. Furthermore, in a recently established *in vivo* human to mouse xeno-transplantation model, human steady-state DCs (and IPCs) develop in relatively normal frequencies, while monocytes only represent a minor cell fraction [80, 81]. Taken together, these data suggest that the "flt3-license pathway" would be the dominant pathway in steady-state DC and IPC development, and that *in vivo* DC development

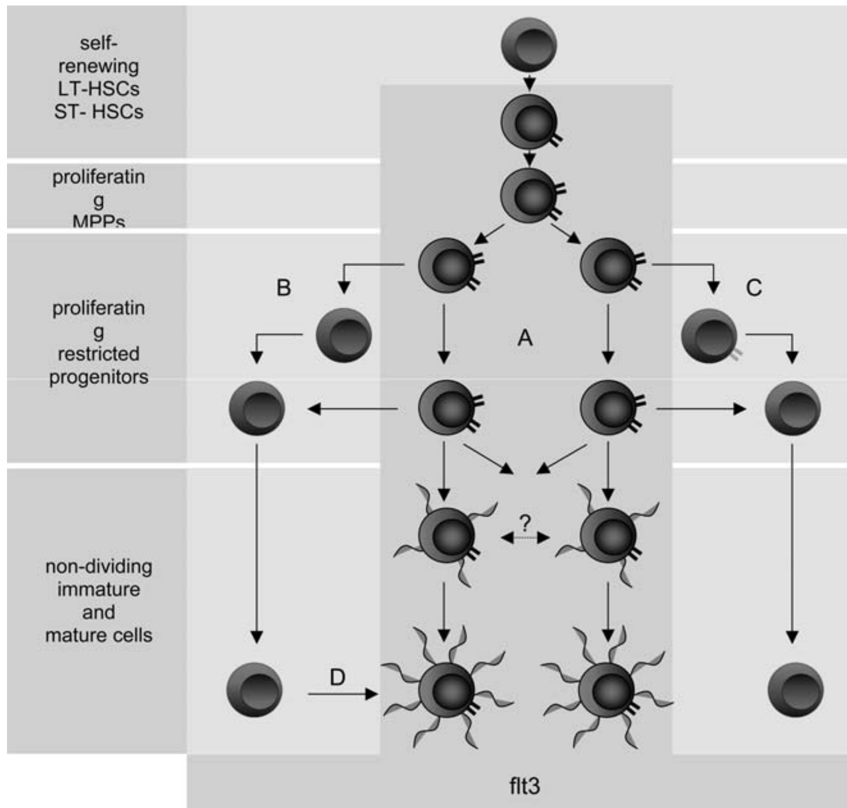

Fig. 2.1 A "flt3-license" working model for steady-state dendritic and natural interferon-producing cell development from early hematopoietic progenitor cells. (A) "flt3-license pathway" for DC and IPC development, (B) flt3 downregulation and loss of DC and IPC developmental options, (C) loss of DC and IPC developmental options in flt3 positive cells due to competitive, dominant lineage-restriction signals, (D) non-steady-state, inflammatory, flt3-independent DC developmental pathway.

from monocytes might be a rare event in noninflammatory, but possibly a frequent, local GM-CSF-driven event in inflammatory settings. This flt3-independent, inflammatory, nonproliferative DC developmental pathway is depicted in Fig. 2.1, pathway D.

2.6 Conclusions

Based on current *in vitro* and *in vivo* data on DC and IPC development, it is reasonable to propose a "flt3-license" working model for steady-state dendritic and natural interferon-producing cell development from early hematopoietic progenitors.

This model integrates flt3$^+$ precursors with DC and IPC developmental capacities, irrespective of their alternative developmental restrictions, and suggests that flt3 signal strength might be the earliest regulator in both DC and IPC development. To understand how full differentiation from HSCs to DCs and IPCs is guided, and to determine whether possibly regulatory feedback loops exist to adapt differentiation upon fluctuating demand in steady-state and inflammation, it will be essential to focus on further DC and IPC development-associated growth-factors, as well as at sequential transcriptional events. This is the subject of subsequent chapters.

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3
Growth Factors

Herbert Strobl, Barbara Platzer, Almut Jörgl, Sabine Taschner, Leonhard Heinz and Peter Reisner

3.1
Introduction

Cytokines critically influence the development of dendritic cells (DC) *in vivo*. Several *in vitro* culture systems have been established to reflect the differentiation of distinct DC subsets under defined cytokine conditions. *Ex vivo* development of DC from purified hematopoietic progenitor cells or monocytes in response to cytokine stimulation typically proceeds over a period of several days. Starting cell populations such as $CD34^+$ hematopoietic progenitor cells are heterogeneous. Therefore these culture systems simultaneously contain cells of various lineages and differentiation stages. Predominance of one cell type over another in culture is modulated in response to different cytokine stimuli. In humans, three distinct DC developmental pathways have been delineated. Two of these, i.e. epithelial Langerhans-type DC (LC) and interstitial/dermal DC (intDC), arise from phenotypically distinct myelomonocytic intermediates, whereas cells of the third DC subset differentiation pathway, i.e. plasmacytoid DC (pDC), may be distantly related to myelomonocytic cells. The two myeloid-related DC subsets can be generated at high purities and yields in response to defined cytokine stimuli. *In vitro* studies of human $CD34^+$ cord blood progenitor/stem cells have been most informative in delineating these three pathways, and in defining their specific cytokine requirements. A hierarchical view of cytokines known to regulate *in vitro* DC development from progenitor cells is shown in Fig. 3.1. Early acting cytokine stimuli such as flt3 ligand (FL) in concert with stem cell factor (SCF) and/or thrombopoietin (TPO) promote expansion of DC progenitor cells. These DC progenitors can be induced to develop along myeloid-related DC intermediates in response to GM-CSF plus TNFα. Certain cytokines such as TGF-β1, IL-4 and IL-15 regulate *in vitro* myeloid DC subset differentiation. As yet undefined factors in serum additionally modulate DC subset specification. Post-committed monocytic intermediates of myeloid DC development have been identified. These cells can be driven (“polarized”) to develop along several DC and monocyte/macrophage subset phenotypes.

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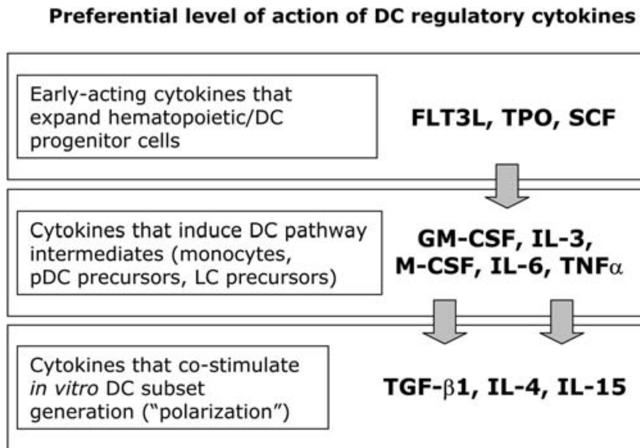

Fig. 3.1 Cytokine regulation of DC development. According to their preferential stage of action within DC hematopoiesis, cytokines may be roughly assigned into three nonexclusive hierarchical groups. (1) early-acting cytokines, (2) cytokines that induce DC differentiation, and (3) cytokines that modulate DC subset differentiation.

The aim of this review is to provide a brief introduction to and overview of DC generation culture models and cytokine signals that regulate DC subset development. These models are well-established in the human system and have been critical for the study of human DC biology with a view toward clinical application in cell therapy. Therefore we predominantly focus on human – rather than murine – DC generation models from either $CD34^+$ hematopoietic progenitor/stem cells or peripheral blood monocytes.

3.2 Short Description of Key Cytokines Involved in DC Development

3.2.1 Flt3 Ligand (FLT3L, FL)

FL is expressed by bone marrow stroma fibroblasts and T cells; it stimulates expansion and differentiation of hematopoietic progenitor and stem cells. [1, 2].

Molecule: Type I transmembrane protein, can also be cleaved to a soluble form, both are biologically active; human and murine FL show cross-species activity (72% amino acid identity) [2].

Receptor: flt3 (fsm-like tyrosine kinase) is a member of receptor tyrosine kinase (RTK) subclass III family; other examples of this receptor family are M-CSF receptor and stem cell factor receptor (c-kit) [3]; flt3 is expressed by early myeloid and lymphoid progenitors, most types of leukemias, but flt3 is not expressed by eryth-

rocytes, megakaryocytes or mast cells [4]; flt3$^+$ murine bone marrow population includes common myeloid progenitors and common lymphoid progenitors and contains precursors of all DC lineages including pDC [5].

Signal transduction: FL binding induces flt3 homodimerization, phosphorylation and internalization; subsequent activation of phosphatidylinositol 3-kinase (PI3-K) and RAS pathways. Stat 3 was shown to be required for FL-dependent DC differentiation [6].

Knock out phenotype: Flt3 deficient mutant mice show relatively normal hematopoiesis, reduced pro-B and pre-B compartments, deficiency in multilineage repopulating function of early progenitors [7]. FL deficient mutant mice display reduced occurrence of various DC subsets; additionally NK, myeloid and B cell subset deficiencies [8, 9].

Function in DC development: In vitro effects of FL on DC are described below. *In vivo* administration of FL in mice results in large increases of DC numbers (including pDC) in various organs, mobilization of pluripotent progenitors into peripheral blood, expansion of immature B cells [10–14]. In humans, *in vivo* administration of FL increases the numbers of circulating myeloid DC and pDC; FL acts synergistically with G-CSF for progenitor cell expansion [13, 14].

3.2.2 GM-CSF (Granulocyte-macrophage Colony-stimulating Factor)

GM-CSF is produced by many cell types including T cells, macrophages, endothelial cells and fibroblasts; it acts on early bone marrow progenitors and promotes proliferation and differentiation of granulocytes, macrophages and DC [15].

Cytokine family: GM-CSF belongs to a common cytokine family with IL-3 and IL-5, encoded on human chromosome 5 (reviewed in: [16] and [17]).

Receptor: common beta chain (βc) with IL-3 and IL-5 receptors, specific α-chain for GM-CSF.

Signal transduction: Activation of the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (-MAPK) cascade pathway (including ERK, JNK and p38 pathways), and the phosphatidylinositol 3-kinase (PI3-K) pathways.

Knock out phenotype: GM-CSF$^{-/-}$ mice show normal hematopoiesis, but abnormalities in the lung, i.e. extensive hyperplasia around the lung airways and veins, reminiscent of pulmonary alveolar proteinosis (PAP) in humans, reduced numbers of alveolar macrophages and lymphoid hyperplasia around lung airways [16].

Function in DC development (human studies, see below): Widely used to generate myeloid DC *in vitro* from murine and human progenitor cells. Inaba et al. showed that GM-CSF induces DC *in vitro* from murine bone marrow [18] and peripheral blood cells [19]; however *in vivo* it is not absolutely required; GM-CSF$^{-/-}$ and GM-CSF-R$^{-/-}$ mice have only minor reductions of DC within lymph nodes, spleen and thymus [20].

3.2.3 Interleukin 3 (IL-3)

IL-3 is a multipotent hematopoietic growth factor produced among other cells by activated T cells, monocytes/macrophages and stroma cells; induces proliferation of progenitor cells of the myeloid [21] as well as of the lymphoid lineages [22]; growth factor for mast cells [23].

Receptor: IL-3 receptor consists of an IL-3Rα subunit and a common beta receptor (βc) shared with GM-CSF and IL-5. In mice, a second β chain has been identified ($\beta c_{IL\text{-}3}$) with redundant function [16].

Signal transduction: see GM-CSF.

Function in DC development: IL-3 is a key cytokine for the proliferation and survival of human plasmacytoid DC [24]. IL-3/IL-4 versus GM-CSF/IL-4 differentially regulate monocyte DC function. Monocyte-derived DC generated in the presence of IL-3/IL-4 show decreased IL-12 production and promote therefore TH2 responses in contrast to DC derived with GM-CSF/IL-4 [25].

3.2.4 Interleukin 4 (IL-4)

IL-4 is multifunctional cytokine produced by different cells of hematopoietic origin, such as T cells, mast cells, and basophils; stimulates the proliferation of B cells, enhances the expression of cell surface molecules such as MHC class II and the low affinity Fc receptor for IgE (CD23); at least in mice, IL-4 is indispensable for the Ig heavy chain class switching to IgE; promotes TH2 differentiation of naïve T cells [26].

Receptor: IL-4 can bind to two types of receptors: Type I heterodimers consist of the IL-4R α-chain and common cytokine receptor γ-chain; the latter is shared by receptors for IL-2, IL-7, IL-9, and IL-15. The type II receptor comprises an IL-4Rα chain in association with the IL-13Rα1 chain.

Signal transduction: IL-4 binding to the IL-4Rα-chain causes association of the γc chain with the IL-4Rα-chain complex followed by the activation of the receptor-associated kinases, Janus kinase-1 (JAK-1) and JAK-3; these kinases activate Stat6 [26].

Knock out phenotype: $IL\text{-}4^{-/-}$ mice have normal numbers of splenic and thymic DC with T-cell stimulatory capacities comparable with wild-type mice [27].

DC development: For human DC generation from monocytes IL-4 is mandatory [28]; its addition to GM-CSF or IL-3 inhibits macrophage outgrowth in these cultures; however in mice high doses of GM-CSF alone are sufficient to generate DC, while IL-4 effects were observed only at low doses of GM-CSF [18]; recently it was found that IL-4 promotes outgrowth of LC-like DC from mouse bone marrow [29].

In addition, DC maturation inducing capacity was described for IL-4 [30]; it was shown that it increases IL-12p70 production of freshly isolated mouse splenic DC, freshly isolated human thymic DC, and cultured human monocyte-derived DC (MoDC) [31]. The IL-4R type II promotes upregulation of MHC class II and co-stimulatory molecules by DC in a GM-CSF dependent manner [30].

3.2.5 Interleukin-15 (IL-15)

IL-15 is a 14–15 kDa cytokine expressed in numerous normal human tissues in a broad range of cell types, including activated monocytes, DC, osteoclasts, fibroblasts, keratinocytes and endothelial cells [32, 33]. It promotes activation of T cells, neutrophils and macrophages and plays a critical role in development, survival, and function of NK cells. IL-15 is also associated with several inflammatory disorders, including rheumatoid arthritis, psoriasis and pulmonary inflammatory diseases [34].

Receptor: The heterotrimeric IL-15 receptor (IL-15R) includes the IL-2Rβ-chain and γ-chain, together with a unique α-chain (IL-15R). IL-15Rα-deficient mice exhibit lymphopenia due to reduced proliferation and homing of mature lymphocytes, particularly of the $CD8^+$ subset.

Signal transduction: Like IL-2, the IL-15Rαβγ complex signals through JAK1/3 and STAT3/5 [34].

Knock out phenotype: Disruption of the common γc chain leads to the absence of lymph nodes and Peyer's patches [35].

Function in DC development: Unlike IL-4, IL-15 skews human monocyte differentiation into DC with LC features [36]. Furthermore IL-15 is important for DC-derived IL-2 production [37].

3.2.6 TNFα (Tumor Necrosis Factor-alpha)

TNFα is implicated in host defense, inflammation, autoimmunity, cell growth, apoptosis, organogenesis, hematopoiesis, and stress response. Associated diseases include rheumatoid arthritis, arteriosclerosis, multiple sclerosis, cancer, osteoporosis and diabetes. TNFα is primarily produced by activated macrophages (reviewed in [38]).

TNF superfamily: 19 members in humans, characterized by the TNF homology domain (THD), membrane bound or soluble (cleaved by proteases), active form: homotrimer.

Receptor: TNF-R1 and TNF-R2, both belong to the TNF receptor superfamily (29 known members in humans, e.g. CD40, fas, CD30, CD27), TNF-R1 is broadly expressed (reviewed in [38]).

Signal transduction: cytoplasmic tail of TNF-R interacts with 2 classes of adapters: TNF-R associated factors (TRAFs) and TNF-R associated death domain (TRADD); downstream signaling: activation of caspase 8 (apoptosis), cIAP-1 (anti-apoptotic), JNK (survival and cell growth), NF-κB (differentiation and maturation) [39].

Knock out phenotype: TNF-R1 k.o.: absent follicular DC and germinal centers, LC present, resistance to LPS, migration intact in TNF-R1 k.o., impaired in TNF-R2 k.o. [40–43]. TNFα deficient mutant mouse: normal DC numbers in lymph nodes and payers patches, Langerhans cells present, migration intact, abnormal follicular DC organization [42, 41].

Function in DC development: required for differentiation from hematopoietic progenitor cells to intDC and LC and maturation [28, 44].

3.2.7
TGF-β1 (Transforming Growth Factor Beta-1)

TGF-β signaling regulates distinct cellular processes, including proliferation, differentiation, extracellular matrix formation, and apoptosis [45, 46]. Among hematopoietic cells positive or negative effect on cell proliferation and differentiation were described [47, 48]. TGF-β family receptors and their ligands are expressed in almost every tissue in the body; autoproduction by many cells including $CD34^+$ progenitors, monocytes and LC [49].

Cytokine family: Member of the large TGF-β receptor superfamily of structurally related proteins, including TGF-β1, 2, 3, activins, Nodal, myostatin, bone morphogenetic proteins (BMPs) and others. TGF-β1, 2, 3 show redundant and nonredundant functions.

Receptor: Functional TGF-β family receptors consist of two "type-II" and two "type-I" transmembrane serine/threonine kinase receptors. Signaling occurs when TGF-β binds to active type-II receptor dimers, which further phosphorylate and recruit type-I dimers, therewith forming a heterotetrameric receptor [50].

Signal transduction: TGF-β family signaling can be Smad-dependent or Smad-independent. Activated type-I receptors phosphorylate and bind receptor-regulated Smads (R-Smad), Smad 2/3, which form a complex with Smad 4 and translocate to the nucleus, regulating transcription of target genes (e.g. Runx proteins, [51]). TGF-β signaling is negatively regulated via the inhibitory Smads 6/7 (e.g. Smad7/-Smurf1-2 interaction). Smad-independent signaling occurs through MAPK, RhoA and TAK1/MEKK1 pathways.

Knock out phenotype: 50% of TGF-β1 deficient mice die because of severe inflammatory responses, indicating essential roles of TGF-β1 in the maintenance of immune homeostasis [52, 53]; lack of LC [54, 55]; reduced numbers of erythroid cells [56]. TGF-β type-II receptor knockout mice have similar phenotype to that of TGF-β1 knockout mice [57].

Function in DC development: (see below).

3.3 Regulation of *in vitro* DC Hematopoiesis by Cooperating Cytokine Signals

3.3.1 Cytokines in the Induction and Amplification of $CD1a^+$ Myeloid DC Subsets from Hematopoietic Progenitor Cells *in vitro*

In 1992 Caux et al. demonstrated that addition of TNFα to GM-CSF-supplemented cultures of purified $CD34^+$ hematopoietic progenitor/stem cells induces a population of $CD1a^+$ DC-like cells with high allo-stimulatory capacity [58]. Subsequent studies demonstrated that two myeloid-related DC subsets, LC and dermal/interstitial DC (intDC), co-develop *in vitro* from $CD34^+$ in response to GM-CSF plus TNFα stimulation [59, 60]. Furthermore, DC subset generation from $CD34^+$ cells was found to be preceded by the development of two distinct precursor ("intermediate") cell populations arising at days 5 to 6 during culture. Cells of the LC pathway arise from $CD1a^+$ $CD14^-$ intermediates. LC are characterized by expression of CD1a, Langerin, E-cadherin, and Birbeck granules. Cells of the second DC pathway arise from $CD14^+$ intermediates. This latter pathway gives rise to germinal center and intDC defined by the expression of CD1a, CD68 and factor XIIIa. Similar to germinal center DC [61], the $CD14^+$ cell-derived population displays activities on B cells, whereas the first subset, i.e. $CD1a^+$-derived LC and *ex vivo* isolated LC, lack these activities [60, 62]. The cytokine combination GM-CSF plus TNFα is most critical for the optimal induction of these two myeloid DC subsets from $CD34^+$ cells. Another class of cytokines known as "early-acting" hematopoietic cytokines cooperate with GM-CSF/TNFα for amplification of myeloid DC in DC generation cultures initiated from hematopoietic progenitor cells. Among these, stem cell factor (SCF) enhances total cell yields without changing percentages of DC generated [63]. Furthermore, SCF increases GM-CSF/TNFα-dependent monocyte/macrophage/DC colony-plating efficiency approximately threefold [64]. Another early-acting cytokine, flt3 ligand (FL), has evolved as a key regulator of DC development *in vitro* and *in vivo*. *In vivo* administration of FL increases yields and percentages of both myeloid and plasmacytoid DC in peripheral blood of healthy adults [13] and mice [65]. *In vitro* experiments revealed that FL approximately doubled percentages of $CD1a^+$ cells (from 30 to 60%) when added to serum-free DC generation cultures supplemented with GM-CSF/TNFα/TGF-β1 with or without SCF [66]. In these cultures, FL seems to enhance the expansion of monocytic/DC precursors (see below). A third early-acting cytokine, thrombopoietin (TPO), efficiently costimulates expansion of immature hematopoitic progenitors together with FL [67], however when directly added to DC generation cultures, TPO failed to enhance total yields of $CD1a^+$ DC [68]. The former two early-acting cytokines, SCF and FL synergistically enhance yields of DC when added together to GM-CSF/-TNFα/TGF-β1 supplemented serum-free cultures [66, 69]. This 5-factor combination substantially induced DC colony-forming activity of $CD34^+$ cells (Fig. 3.2). Furthermore, this combination selectively induces LC differentiation (>50% LC) along with a 50–100-fold amplification of total cell numbers within 7 to 10 days af-

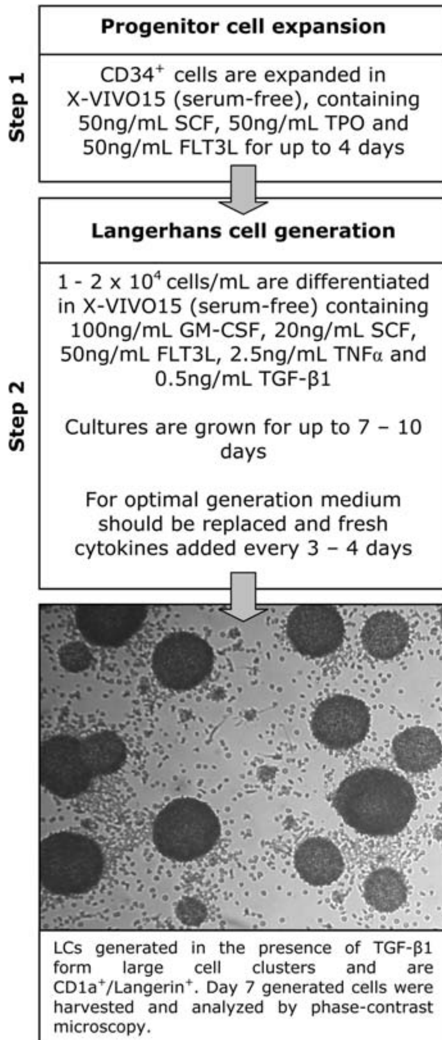

Fig. 3.2 LC generation protocol from $CD34^+$ hematopoietic progenitor cells. $CD34^+$ cells from cord blood or G-CSF mobilized peripheral blood can be induced to differentiate into LCs in serum-free medium. These LC generation cultures are supplemented with the cytokines GM-CSF, TNFα, SCF, FL and TGF-β1. LC are recognized morphologically by typical TGF-β1-dependent homotypic LC cluster formation. A pre-expansion step may be added for manipulating LC (e.g. for optimal cell cycling as required for efficient retroviral gene transfer). According to our experiences pre-expansion of cells beyond day 4 decreases the percentages of LCs in subsequent LC generation cultures.

ter culture initiation using $CD34^+$ progenitor cells isolated from cord blood [66] or from G-CSF mobilized adult peripheral blood [70, 71]. Serum-free generated LC induced by this cytokine combination form typical homotypic cell clusters (see Fig. 3.2; an effect dependent on TGF-β1) enabling purification of $CD1a^+$ LC to >85% by using a simple 1-g sedimentation step [71, 72]. In conclusion, early-acting cytokines (FL and SCF) cooperate with DC-inducing cytokines (GM-CSF, TNFα, TGF-β1) for the generation of DC in cultures from $CD34^+$ hematopoietic progenitor cells, and these cultures support efficient LC generation in serum-free medium. Fig. 3.2 gives an example of such a DC generation protocol from $CD34^+$ progenitor cells.

3.3.2 Cytokines for Pre-expansion of Myeloid DC Progenitor Cells

The cytokine combinations containing GM-CSF/TNFα, described above, efficiently induce myeloid DC differentiation from $CD34^+$ hematopoietic progenitor cells. However, they fail to simultaneously support DC progenitor cell expansion. Furthermore, GM-CSF plus IL-4, a cytokine combination for the generation of $CD1a^+$ DC from peripheral blood monocytes ("monocyte-derived" DC, see below) fails to promote DC generation from $CD34^+$ cells. Therefore, two-step culture models have been established with the aim of pre-expanding cytokine-responsive DC precursor cells. These culture systems comprise a combination of early-acting hematopoietic cytokines for pre-expansion of DC progenitors prior to induction of DC using specific cytokine conditions such as GM-CSF plus IL-4. To monitor *ex vivo* DC progenitor cell expansion various assay systems have been applied. Purified $CD34^+$ cells from either cord blood or G-CSF mobilized adult blood were used for such pre-expansion experiments. Based on the observations that TPO and FL synergize in long-term hematopoietic progenitor cell expansion [67] and initial observations that FL/TPO or FL/TPO/SCF promote DC progenitor cell expansion [73], 3-factor combinations comprising the early-acting cytokines FL/TPO/SCF have recently evolved for the pre-expansion of myeloid DC progenitors. These cytokines effectively expanded GM-CSF/IL-4 responsive DC progenitors. For instance, in two studies FL/TPO/SCF supported a 245-fold increase of total cellularity of $CD34^+$ cord blood cells [74] or 38-fold expansion of G-CSF mobilized $CD34^+$ cells [75] within 4 weeks, in either serum-free or serum-supplemented medium, respectively. These cultures supported the generation of cells that possessed $CD1a^+$ DC differentiation potential in subsequent GM-CSF/IL-4 assay cultures (>50% $CD1a^+$ cells). In other studies, such FL/TPO/SCF supplemented serum-free pre-expansion cultures of $CD34^+$ cord blood progenitor cells were further supplemented with IL-6 [76] or an IL-6 receptor/IL-6 fusion protein (hyper-IL-6) that directly activates gp130 receptors [77–80]. These conditions led to on average 12.7-fold (day 7, [76]) or 100-fold (day 12 to 14, [79]) amplification of GM-CSF/IL-4 responsive DC progenitors, respectively. Hyper-IL6 has previously been shown to expand immature human hematopoietic progenitors capable of reconstituting immunodeficient mice [81]. In addition, IL-6 upregulates M-CSF receptors and thus M-CSF responsiveness of monocytic cells, and redirects DC towards macrophages [82, 83]. This effect seems to contribute to $CD14^+$ monocytic cell differentiation in FL/TPO/SCF progenitor cell expansion cultures ([76] and B. Platzer, unpublished observations). Compared with IL-6, addition of the cytokine IL-3 (to these FL/SCF/TPO cultures) approximately doubled d7 DC progenitor yields in serum-free medium and favored generation of $CD1a^+$ over $CD14^+$ progeny [76]. Interestingly, very high expansion values were described using a sequential pre-expansion protocol [75]. In this protocol $CD34^+$ cells from G-CSF mobilized blood were first stimulated with SCF/FL/IL-3/IL-6 for 3 weeks, followed by an additional week in FL/SCF/TPO, resulting in a 453-fold increase in total cell numbers. Expanded cells efficiently gave rise to $CD1a^+$ DC after GM-CSF/IL4 induction (41.3%, [75]). Percentages of DC

dropped from 41.3% to 19% CD1a+ cells when cells were continuously cultured for 4 weeks in SCF/FL/IL-3/IL-6; conversely cell yields but not percentages of CD1a+ DC dropped (from 453-fold to 38-fold expansion) in FL/SCF/TPO 4-week continuous cultures. Thus, current protocols allow substantial pre-expansion of GM-CSF/IL-4-responsive "monocyte-derived" DC for extended culture periods. GM-CSF plus IL-4 preferentially induce DC differentiation from monocytic cells, but this cytokine combination fails to support immature DC progenitor cell growth. Therefore, studies are of interest which directly assay for colony-forming unit (CFU)-DC in semisolid methyl cellulose assays in response to the above-mentioned GM-CSF plus TNFα and SCF cytokine mix. Curti et al. [84] demonstrated that indeed such CFU-DC can be expanded (100-fold) *in vitro* in response to SCF and FL from CD34+ immature progenitor cells. Expansion values might differ for precursors of distinct DC subsets. We observed that GM-CSF/TNFα/TGF-β1-responsive LC precursors generated in serum-free medium cannot be expanded beyond week 1 in FL/SCF/TPO cultures of CD34+ cord blood cells (unpublished observations). This might be consistent with derivation of LC from early myelomonocytic precursors (see below and [85]).

3.3.3 Cytokine Combinations that Promote Myeloid DC Expansion Reciprocally Inhibit Plasmacytoid DC Development in Suspension Cultures

Plasmacytoid DC, first described by Grouard et al. [24], are widely recognized as a third independent DC sublineage pathway arising from hematopoietic progenitor/stem cells both in human and murine systems (Fig. 3.3). A detailed description of pDC is provided elsewhere in this book (see Chapter 13). pDC precursors show similarities to, as well as differences from, myeloid-related (also termed "conventional") DC with regards to cytokine responsiveness and lineage marker expression. Human pDC express low levels of the myelomonocyte surface antigens CD33 and CD13 [24] and lack the highly lineage specific intracellular myeloid proteins myeloperoxidase (MPO) and lysozyme used for myeloid lineage assignment of undifferentiated leukemia blast cells [86]. All these myeloid lineage marker molecules are expressed by peripheral blood monocytes [87] and most of them (with the exception of MPO) are expressed by CD11c+ myeloid-related blood DC precursors *in vivo* [86, 88]. In line with this pDC precursors differ from myelomonopoietic cells in their *in vitro* cytokine responsiveness. First, unlike myeloid DC precursors, circulating or tonsil pDC precursors fail to respond to GM-CSF. In contrast they express high levels of IL-3 receptor alpha chain (CD123) [89–91] and respond to IL-3 *in vitro* [24, 86]. IL-3 allows *in vitro* culture of pDC involving infrequent cycling of pDC, however without concomitant increases in pDC cell numbers. Furthermore, IL3 alone induces a DC-like cell morphology by freshly isolated round-shaped pDC precursors [24, 86]. A second major difference between pDC and myeloid DC progenitors is evident from pDC induction experiments from CD34+ cells. Both pDC and myeloid DC are positively stimulated by FL and in the murine system FL induces very high percentages (45%) and high yields of pDC from bone marrow cells

within 10 days [92]. In contrast, pDC generation is inhibited by any of the other costimulatory cytokines that promote myelopoiesis or myeloid DC generation in suspension cultures (human: SCF, GM-CSF, G-CSF, IL-3 [93]; murine: GM-CSF, TNFα, [92]). FL seems to be most critical for pDC generation from $CD34^+$ cells and addition of TPO strongly enhances total pDC yields but not pDC percentages. Interestingly, optimal pDC development from $CD34^+$ cells in FL or FL/TPO suspension cultures is seen only late in culture, beyond 2 to 3 weeks after culture initiation [94]. Furthermore, even under optimized culture conditions a human pDC phenotype is infrequently observed in these cultures (i.e. approximately 3–4%, compared with >60% for $CD1a^+$ “conventional” myeloid DC). A recent study found that IL-3 addition to FL/SCF/TPO in serum-free medium promotes the generation of $CD13^-$ pDC precursors from $CD34^+$ cells after 7 days of culture [76]. More rapid generation of pDC was reported when stroma contact cultures (murine S17 stroma cells) instead of suspension culture models were used. For instance, approximately 1–15% of cells generated from human fetal liver $CD34^+CD38^-$ cells ([95]), or 5–10% generated from thymic $CD34^+CD1a^-$ cells [96] represented pDC within 5 - days in these co-culture systems. These pDC generated *in vitro* phenotypically and functionally resemble pDC isolated from peripheral blood (identified as $CD11c^-HLA\text{-}DR^+CD123^{bright}CD1a^-$ cells; they show typical plasmacytoid cell morphology, high IFNα production capacity and similar Toll-like receptor (TLR) expression pattern). However, their exact lineage relationship to co-generated myelomonocytic cells and “conventional” DC is poorly defined. Several observations may be compatible with a common myeloid origin of pDC and monocytic cells. In FL or FL plus TPO containing cultures, infrequent pDC are admixed to high percentages of monocytic cells [94], an observation that might potentially indicate that monocytes and pDC share common precursors. Karsunky et al. [97] recently showed that common myeloid progenitor cells (CMP) and to a lesser extent granulomonocyte progenitors (GMP) or common lymphoid progenitors (CLP) give rise to pDC upon transplantation into irradiated murine recipients. Furthermore, pDC can be generated *in vitro* from CMP in the presence of FL, whereas common lymphoid progenitors (CLPs) require additional survival signals such as IL7 plus SCF or transgenic bcl-2 [97]. Earlier studies demonstrated that human pDC arise *in vitro* from purified $CD123^{hi}M\text{-}CSFR^{lo}$ intermediates in cultures of $M\text{-}CSFR^{hi}CD34^+CD123^{lo}$ granulomonocytic bone marrow progenitor cells [89]. Furthermore, in contrast to low/absent expression of above mentioned myeloid lineage antigens, pDC share bright intracellular CD68 expression with macrophages and promyelocytic cells [86, 98]. In fact plasmacytoid “T cells” originally identified in 1975 by Lennert [99], by their plasmacytoid ultrastructure, were renamed in 1986 as plasmacytoid “monocytes” based on bright CD68 immunohistology staining [100]. Additionally, pDC generated *in vitro* from thymic $CD34^+CD1a^-$ cells in the presence of S17 stroma cells convert to myeloid-type DC associated with upregulation of the myeloid DC affiliated transcription factors PU.1 and RelB [96]. These findings might correspond to recent observations in the murine system showing that bone marrow pDC identified as $B220^+GR1^+CD11c^+$ DC acquire myeloid DC phenotype (neo-expression of CD11b, loss of B220) *in vivo* in response to LCMV infection [101].

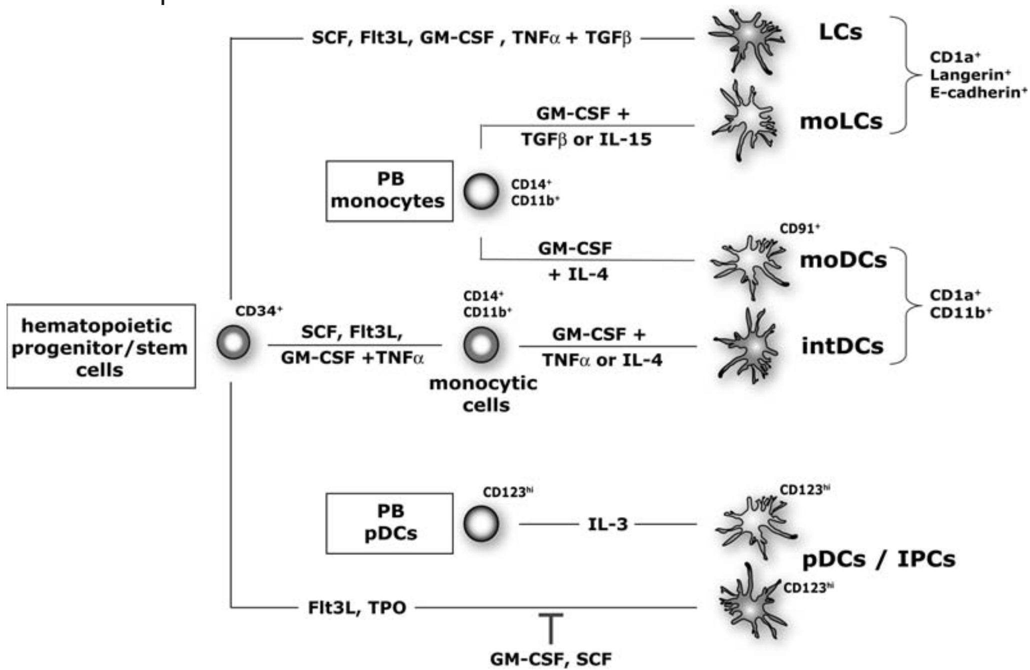

Fig. 3.3 Overview of cytokine signals that direct differentiation of defined human DC subsets from precursors *in vitro*. Precursor pathways of at least three separate DC subset pathways can be delineated (LC, intDC, pDC). Distinct cytokine combinations differentially induce CD34$^+$ human hematopoietic progenitor cells to develop along these three DC subset pathways. Alternatively, CD14$^+$CD11b$^+$ peripheral blood monocytes can be induced to develop along LC or intDC pathways in response to the cytokine signals shown. CD11c$^-$/CD123$^+$/lin$^-$ circulating pDC precursors require IL-3 for survival and maintenance *in vitro*. pDC are also known as interferon-producing cells (IPC).

These "converted" pDC progeny still showed molecular markers of pDC, and this effect was dependent on expression of type 1 interferon receptor. Thus a picture emerges suggesting that initial differentiation stages of pDC and monocytic cells are shared and that pDC precursors in bone marrow may still be capable of differentiating into myeloid DC in response to type I interferons. A better characterization of pDC developmental stages beyond the CD34$^+$ progenitor cell stage *in vivo* may help to clarify the lineage relationship of these cells to classical myelomonocytic cells and may provide a basis for improved generation of pDC from progenitor cells in suspension cultures *in vitro*.

3.3.4 TGF-β1 and its Essential Co-signals for LC Differentiation from Hematopoietic Progenitor/Stem Cells

It has become evident that the pleotropic cytokine TGF-β1 is critical for LC development [48]. We identified TGF-β1 as a key factor for *in vitro* LC differentiation when we attempted to replace exogenous serum from GM-CSF/TNF/SCF-containing (then the “standard”) DC generation cultures of $CD34^+$ cord blood cells [102]. Omission of 10% plasma or FCS from these cultures led to profound inhibition of DC development. Addition of TNFα to serum-free GM-CSF/SCF-stimulated $CD34^+$ progenitor cell cultures induced apoptosis by a portion of DC progenitors. Addition of TGF-β1 (which occurs abundantly in serum) protected DC precursors from undergoing apoptosis [103]; the resultant DC population induced by TGF-β1 resembled LC as revealed from their typical phenotype ($CD1a^{bright}$, $Langerin^+$) and by numerous intracellular Birbeck granules [102]. In line with *in vitro* dependency of LC generation on TGF-β1 signaling, TGF-β1 deficient mutant mice lack LC [54]. Furthermore, TGF-β1 from autocrine (LC themselves) or paracrine sources (other skin cells) is sufficient for LC differentiation *in vivo* [104]. Epithelial/epidermal tissues are abundant sources of TGF-β1, with keratinocytes expressing low levels whereas LC expressing high levels of immunoreactive TGF-β1 [49]. More detailed analyses of the mechanisms involved in TGF-β1-dependent LC generation revealed that TGF-β1 has to be added early at day 1 to these cultures of $CD34^+$ cells for optimal LC generation. In line with this early stimulatory effect of TGF-β1, anti-TGF-β1 antibody addition to $CD34^+$ cells at culture onset abrogated day 12 $CD1a^+$ DC generation and led to lower cell proliferation. This indicates that endogenous TGF-β1 in the cultures contributes to LC differentiation [105]. Mechanistically, in this model TGF-β1 does not seem to act by recruiting an additional progenitor cell subset into DC differentiation, nor by enhancing proliferation [103]. Rather, TGF-β redirects monocytic cells to LC differentiation. Under suboptimal cytokine co-stimulation (without FL) TGF-β1-mediated protection of LC precursor apoptosis (as mentioned above) seems to contribute additionally to LC differentiation [103]. These positive stimulatory effects of TGF-β1 on LC differentiation were found to be critically dependent on the simultaneous presence of the cytokines GM-CSF plus TNFα in culture [102, 103]. In the absence of TNFα exogenous TGF-β1 reduced GM-CSF/SCF-induced progenitor cell proliferation, which is in line with the well-documented negative effects of exogenous TGF-β1 or endogenous (autocrine) TGF-β1 on hematopoietic progenitor cell proliferation [106, 107]. In conclusion, the positive stimulatory effects of TGF-β1 on LC induction from $CD34^+$ cells critically depend on the differentiation stage of myelomonocytic progenitors (i.e. early) and on the nature of co-signals (i.e. GM-CSF plus TNFα) simultaneously present in culture.

3.3.5
TGF-β1 Induces LC Differentiation from Monocyte Intermediates

In vitro cytokine stimulation experiments support a common origination of LC and monocytes from $CD34^+$ progenitor cells. Firstly, time kinetics experiments revealed that TGF-β1-dependent LC differentiation involves pregeneration of cells showing (pro-) monocyte marker characteristics (lysozyme, [102]). In turn with induction of LC characteristics (pro-)monocyte characteristics are lost at later time points upon TGF-β1 stimulation [102]. Secondly, FL addition cooperates with TGF-β1 during LC differentiation, and this cooperation seems to result from increased recruitment and/or enhanced proliferation of monopoietic precursors by FL. This is evident from the observations that FL addition doubles percentages of generated LC (from approximately 30 to 60%) in progenitor cell cultures, and that omission of exogenous TGF-β1 from an optimized FL containing 5-factor combination (see above) abrogates LC differentiation [66]; instead these cells acquire myelomonocytic features [66]. Therefore in the absence of TGF-β1, FL addition to $CD34^+$ cells promotes myelomonocyte development, and TGF-β1 addition represses early monopoietic features (lysozyme, CD14) reciprocal with the induction of LC lineage characteristics. This phenomenon was similarly seen at the clonogenic progenitor cell level. TGF-β1 addition to single cell cultures of $CD34^+$ cells induced LC colony formation at the expense of larger growing myelomonocytic colonies generated in the absence of TGF-β1. From the overall high clonogenic capacity of $CD34^+$ cord blood progenitors in the presence of GM-CSF, SCF, FL, TNFα with or without TGF-β1 it became evident that TGF-β1 acts on (myelo)-monocytic progenitors to shift their differentiation toward an LC phenotype [66]. These early studies on cytokine co-signaling effects were in line with subsequent studies that isolated putative M/LC precursors from $CD34^+$ progenitor cell cultures. $CD14^+$ early monocytic intermediates arising at days 5 to 6 in "standard" serum-supplemented cultures (i.e. containing GM-CSF+TNFα+SCF plus 10% serum) possess LC differentiation potential in the presence of TGF-β1. However, at later time points, monocytic cells lose this LC differentiation potential [105]. A phenotypic branching point at which monopoietic cells start to lose TGF-β1-dependent LC differentiation potential was identified at the transition of $CD14^+CD11b^-$ early monocytic cells to later $CD14^+CD11b^+$ monocytes [109]. In conclusion, data from $CD34^+$ differentiation models revealed that initial developmental stages of monocytes and LC are shared, but M/LC precursor capacity seems to be lost rapidly during culture of $CD34^+$ cells. Once monocytes acquire a "late" phenotype ($CD14^+CD11b^+$), cells obviously lose TGF-β1-responsive LC differentiation potential. However, these *in vitro* generated $CD14^+CD11b^+$ monocytes still possess intDC potential and multilineage monocyte/macrophage potential.

3.3.6 Redundancy among Cytokine Signals Directing LC Differentiation from CD34⁺ Hematopoietic Progenitor Cells

As mentioned above TGF-β1 is critical for LC differentiation *in vitro* and *in vivo*, and $CD34^+$ progenitors require a cooperation of TGF-β1 plus GM-CSF and TNFα cytokine activities for LC differentiation induction. Are GM-CSF/TNFα essential for LC differentiation induction or might these cytokine signals be replaced by other stimuli? It was shown that GM-CSF can be replaced by IL-3 [110] or by M-CSF [111], still allowing TGF-β1-dependent LC generation from $CD34^+$ cells. However, under these conditions, yields and percentages of LC drop. TNFα enhances TGF-β1-dependent LC differentiation; nevertheless, TGF-β1 seems to be sufficient to induce LC from human $CD34^+$ cells in the absence of TNFα (unpublished observations). Similarly, immature c-kit^+lin^- murine bone marrow progenitor cells can be induced by GM-CSF, FL, SCF and TGF-β1 to develop into E-$cadherin^+$ LC-like cells in the absence of TNFα [112]. Our finding that blockage of the TNFα pathway by retroviral expression of a degradation resistant IκBα molecule does not abrogate initial LC differentiation from human $CD34^+$ progenitor cells is in line with these observations [72]. Therefore, initial LC commitment and development is critically dependent on TGF-β1. In addition, this DC subset may differ from other myeloid-related DC in their dependency on TNFα-mediated NF-κB activation [72, 113].

3.3.7 TGF-β1-dependent LC Induction by in vivo Occurring Candidate Precursors

Do putative LC progenitors *in vivo* similarly respond to above cytokine stimuli essential for LC generation from $CD34^+$ hematopoietic progenitor cells? Murine data suggest that steady state renewal of epidermal LC can be maintained without a continuous influx of bone marrow derived progenitor cells [114]. However, bone marrow progenitors do contribute to the pool of epidermal LC *in vivo* after UV irradiation [114] or after human allogeneic bone marrow transplantation [115]. $CD34^+$ progenitor cell-based LC differentiation models identified a $CD11b^-$ early monopoietic precursor giving rise to LC in response to TGF-β1, suggesting that LC arise from a $CD11b^-$ progenitor cell pathway. Indeed, *in vivo* occurring human LC lack CD11b. Furthermore, TGF-β1 responsive $CD11c^+$ peripheral blood myeloid DC/LC precursors similarly lack CD11b [116]. However, arguing against derivation of LC from a $CD11b^-$ pathway, both $CD11b^+$ peripheral blood monocytes (see below) and $CD11b^+$ dermal LC precursors isolated after 72 h in emigration cultures [117] can be induced by TGF-β1 to acquire (at least certain) LC characteristics. Therefore, an alternative model puts the $CD14^+CD11b^+$ peripheral blood monocyte into the center of both LC and intDC developmental pathways.

3.3.8 Cytokines Promoting the Generation of Monocyte-derived DC

GM-CSF with or without IL-4 induce CD1 molecules by peripheral blood monocytes [118, 119]. Addition of IL4 to GM-CSF represses monocyte features and stably induces $CD1a^+$ DC characteristics by peripheral blood monocytes, an effect occurring in the absence of cell cycling [28]. However, GM-CSF/IL4-generated monocyte-derived DC retain CD11b as well as other marker characteristics of myelomonocytic cells such as MPO protein [120]. These molecules (MPO, CD11b) are absent from the two known major human peripheral blood DC populations *in vivo* [86]. It was then shown that addition of TGF-β1 to GM-CSF/IL-4-containing cultures of monocytes led to upregulation of E-cadherin, Lag-antigen, and to the induction of Birbeck granules [121], indicating that monocytes are "polarized" by these stimuli to adopt LC characteristics. More recently, it was shown that IL-4 seems to counteract, rather than promote TGF-β1-dependent LC subset polarization, thus potentially allowing more specific LC induction from monocytes in the absence of IL-4 [122]. Similar to TGF-β1 co-signaling, IL-15 addition to GM-CSF-supplemented cultures of peripheral blood monocytes resulted in E-cadherin and CD207 upregulation, as well as in CD11b downregulation, consistent with phenotypic conversion to LC [36]. Interestingly, these latter IL-15-induced LC-like cells lacked demonstrable intracellular Birbeck granules [36]. Based on these data it can be envisioned that monocytes are recruited to epidermis or to other epithelial/mucosal tissues and that upon encounter of various microenvironmental signals these cells undergo full phenotypic conversion to LC. *In vitro* modeling of this M to LC transition might require improvement of current culture models for DC generation from monocytes (e.g. as yet to be identified TGF-β1 co-signals might be required for full phenotypic conversion of monocytes to LC *in vitro*). In this regard it is interesting that certain critical DC markers might be "aberrantly" expressed by *in vitro* generated monocyte-derived DC. For instance LC *in vivo* [123] and LC generated from $CD34^+$ cells *in vitro* in response to TGF-β1 ([123] and our own observations) lack DC-SIGN, whereas monocyte-derived DC express high levels of DC-SIGN in response to GM-CSF/IL-4 [123, 124]. In line with the antagonism described above, TGF-β1 versus IL-4 differentially modulate DC-SIGN expression by monocytes [124]. Monocytes might provide an "emergency pathway" for LC-like cell regeneration in addition to reconstitute DC under steady state conditions. For instance, atopic dermatitis lesions contain the so called "inflammatory dendritic epidermal cells" (IDECs) which unlike normal LC co-express both CD11b and CD1a. These cells might represent recent immigrants of $CD14^+CD11b^+$ monocytes [125, 126]. Interestingly, aberrant CD11b expression is also observed in Langerhans cell histiocytosis, a pathologic proliferation of LC of unclear pathogenesis [127, 128].

3.3.9
Evidence for Cellular Heterogeneity of DC Arising from CD34$^+$ Progenitors or Monocytes

Both monocyte-derived DC (see above) and intDC in progenitor cell cultures [59, 60, 63] arise from CD14$^+$CD11b$^+$ cells. Therefore it might be assumed that these DC are very similar if not identical (see Fig. 3.3). Recently a direct comparison of these two DC populations induced by GM-CSF plus IL-4 was performed (i.e. from monocytes versus CD34$^+$ cells). Interestingly, both DC subsets do show many similarities in their surface molecule expression pattern and cytokine production pattern upon stimulation. However, important differences were observed. For instance, only peripheral blood monocyte-derived DC, but not progenitor cell derived DC expressed CD91 [129], and only the former were capable of secreting the bioactive form of IL-12p70 [129, 130]. Furthermore, mono-DC differed in this respect from freshly isolated or *in vitro*-derived LC generated from CD34$^+$ cells [129] or from monocytes [131].

3.4
Conclusions

Based on the identification of a number of key cytokine regulators of DC hematopoiesis and existing detailed information on their downstream signaling pathways and stages of activity during development, optimized protocols have become available for the *in vitro* generation of defined DC subsets from their precursors. However, for application in clinically-oriented studies culture systems are desirable that allow the generation of large numbers and high purities of well-characterized DC under defined serum-free conditions. Recent progress enabled the *in vitro* generation of LC in serum-free cultures from human CD34$^+$ progenitor cells. Similar culture models for the generation of large percentages of human pDC are still lacking. Future studies should intensify optimization of cytokine growth conditions and upscaling of current culture systems for the selective generation of LC, pDC or intDC in clinically applicable defined growth media. Nevertheless, all three DC subsets can now be generated from human progenitor or precursor cells using already published growth conditions. Such *ex vivo* generated DC subsets can be used as models to better characterize functional differences between these DC subsets.

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4
Transcription Factors: Deciphering the Transcription Factor Network of Dendritic Cell Development

Thomas Hieronymus and Martin Zenke

4.1
Introduction

Research over the past several years established a determining role of dendritic cells (DC) in antigen specific immune responses and the maintenance of immunological tolerance. DC occur throughout the organism in both lymphoid and non-lymphoid tissues and constitute a complex system of heterogeneous cells that differ in tissue distribution, surface phenotype and specific functions [1, 2]. It is now assumed that different DC subsets are derived from different hematopoietic lineages [3–5]. However, their precise lineage origin and the underlying molecular mechanisms that determine DC development have remained largely unclear or highly controversial [5, 6].

DC are now believed to originate from both lymphoid and myeloid precursors. Although a definitive model of DC development still remains to be established, recent data, mostly from *in vivo* DC reconstitution assays and the analysis of genetically modified mice, have substantially contributed to our understanding of DC origin. These data have revealed a considerable plasticity of DC development.

DC, like all mature blood cells, develop from a population of multipotent hematopoietic stem cells (HSC), which due to their sustained self-renewal capacity maintain hematopoiesis throughout life. Stem cells and multipotent progenitor cells exhibit a broad gene expression repertoire and promiscuously express genes of several lineages but at low levels [7–10; and references therein]. During development of the different hematopoietic lineages the gene expression repertoire and the options of stem cells and multipotent progenitors become increasingly restricted, leading to the establishment to a specific lineage from the choice of several. Among these more restricted progenitor cells are the well-studied common lymphoid progenitor (CLP) and common myeloid progenitor (CMP; [7–9]). CLP can develop into B cells, T cells, NK cells and lymphoid-derived DC, but not myeloid cells; CMP develop into all myeloid cells, including macrophages, granulocytes and myeloid-derived DC.

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Recent studies challenge this currently prevailing model of a strict separation into CLP and CMP as the first lineage commitment step in adult hematopoiesis [11]. It is rather suggested that the first lineage restriction site is the separation of cells with erythroid/megakaryocytic potential from cells with a lymphoid/myeloid potential. Thus, it remains open whether further so far not recognized restriction sites exist that also determine DC and DC subset development.

Stem/progenitor cell renewal and cell fate choice in the hematopoietic system are regulated by various signaling pathways, including cytokines and cytokine receptors, Wnt and Notch factors [7, 9, 12–14]. These signaling pathways modulate gene activity of lineage determining transcription factors. Transcription factors were demonstrated to play a central role in lineage commitment and differentiation, and factors that control the development of red blood cells, macrophages, B cells and T cells, have been particularly well studied [8, 9, 15]. In contrast, our understanding of the developmental pathways leading to DC is more limited.

Considerable progress has been made over the past years in determining the key molecules that regulate commitment and differentiation of DC from mouse bone marrow, including various transcription factors, cytokines and their receptors. However, their roles in the precise stages, surface phenotypes and lineage restrictions observed early in DC development have remained controversial. In this article we review a panel of transcriptional regulators that were demonstrated to be important for DC development.

4.2 Ikaros

Ikaros is a member of the Krüppel family of zinc finger DNA binding proteins that play a major role for the development of all classes of lymphocytes [16, 17]. The *Ikaros* gene encodes a family of proteins generated by alternative splicing, that share a common *C*-terminal zinc finger domain mediating homo- and heterodimeric interactions with other family members. The *Ikaros* isoforms Ik-1, Ik-2, Ik-3 and Ik-4 contain a second *N*-terminal zinc finger domain is required for sequence-specific DNA binding. The *Ikaros* isoforms, Ik-5, Ik-6, and Ik-7 lack the *N*-terminal zinc finger DNA binding domain. As a consequence, these isoforms can regulate the activity of the DNA binding *Ikaros* proteins in a dominant negative fashion [18, 19]. To determine the role of *Ikaros* in hematopoiesis, the *Ikaros* locus was targeted by two distinct mutations.

Mice homozygous for a deletion in the last translated *Ikaros* exon encoding the *C*-terminal zinc finger are deficient for any functional *Ikaros* protein [17] (referred to as *Ikaros* null mice or *Ikaros* $C^{-/-}$ mice). *Ikaros* $C^{-/-}$ mice lack NK cells and both fetal and adult mature B cells as well as the earliest defined B lineage precursors. Fetally derived T cells and their precursors are also absent. However, postnatally, a severely reduced number (10–30-fold reduction) of T-cell precursors appear in the thymus that undergo abnormal differentiation along the $CD4^+$ T cell pathway and produce severely reduced numbers of γ/δ T cells [17]. Similarly, $CD8\alpha^+$ $Dec205^+$

thymic DC are present in the thymus of *Ikaros* $C^{-/-}$ mice but at much reduced numbers (15-fold reduction). The CD8α$^+$ Dec205$^+$ DC are also present at reduced levels in the spleen, whereas the CD8α$^-$ DC population is completely absent [20].

A distinct mutation in the *N*-terminal DNA binding domain of *Ikaros* results in a truncated protein with impaired sequence specific DNA binding but capable of dimerization with wild type DNA binding *Ikaro*s isoforms and with other *Ikaros* family members and therefore acts in a dominant negative fashion [19, 21, 22]. Consistent with these *in vitro* observations, mice homozygous for the *Ikaros* dominant negative mutation (*Ikaros* $DN^{-/-}$) display more severe lymphoid defects relative to *Ikaros* $C^{-/-}$ mice as *Ikaros* $DN^{-/-}$ mice lack all cells of lymphoid origin [16]. In addition, CD8α$^+$ Dec205$^+$ thymic DC are completely absent in the thymus of *Ikaros* $DN^{-/-}$ mice. Although *Ikaros* $DN^{-/-}$ mice abundantly produce myeloid cells and have enlarged spleen, both the CD8α$^+$ and the CD8α$^-$ DC subtypes are absent [20]. In contrast to the lack of both main conventional DC subtypes, *Ikaros* $DN^{-/-}$ mice possess normal numbers of MHC class II$^+$ epidermal Langerhans cells (LC) [16]. Bone marrow transplantation experiments using both mutant mouse models revealed that the selective defects in DC development of *Ikaros* mutant cells are autonomous and not environmentally determined [20].

The more severe defects in lymphoid and DC development in the *Ikaros* $DN^{-/-}$ mice compared to the *Ikaros* $C^{-/-}$ mice reflect the combined effect of loss of *Ikaros* activity and negative interference by *Ikaros* mutant isoforms towards related factors present in early precursors. In addition to the developmental effects for mature immune cells, some defects in the hematopoietic progenitor and stem cell compartment in both mutant mouse models were described [23]. The effects of the *Ikaros* mutations on HSC activity are apparent in long-term repopulation activity, which is reduced by 20–40-fold in *Ikaros* $C^{-/-}$ mutants and is lost in *Ikaros* $DN^{-/-}$ mice. The reduction in HSC activity correlates well with the progressively reduced levels of the tyrosine kinase receptor c-kit at the cell surface of *Ikaros* $C^{-/-}$ and $DN^{-/-}$ hematopoietic progenitor cells. Even more interesting in respect to DC development is the observation that hematopoietic progenitor cells from both *Ikaros* mutations lack expression of the receptor tyrosine kinase Flt3 [23].

4.3 RelB

NF-κB has been demonstrated to play a critical role in regulating the development and function of DC. Early evidence came from the observation that RelB is highly expressed in interdigitating DC of the thymic medulla and the deep cortex of lymph nodes [24]. Furthermore, when chicken bone marrow cells were infected with a retrovirus carrying a conditional v-rel allele, a DC progenitor was transformed. Inactivation of the transforming oncogene resulted in the maturation of the progenitors to mature antigen-presenting cells [25].

Consistent with a function of NF-κB in DC maturation, several groups have reported an increase in NF-κB expression and nuclear activity in primary DC and an

immortalized DC line upon *in vitro* differentiation [26–29]. Differentiation of this cell line to antigen presenting cells can be efficiently blocked by treatment with TPCK, a serine protease inhibitor known to prevent NF-κB activation [30]. However, TPCK has pleiotropic effects and is not a specific NF-κB inhibitor. Therefore, the conclusions drawn have to be corroborated by more specific inhibitors.

The most convincing evidence for a critical role of NF-κB in the differentiation process came from the analysis of knockout animals. $RelB^{-/-}$ mice lack DC in both thymus and spleen, but they possess normal frequencies of LC [31, 32]. It was also shown that RelB affects $CD8\alpha^-$ DC but not $CD8\alpha^+$ DC. In $RelB^{-/-}$ mice numbers of $CD8\alpha^-$ DC are reduced due to the disrupted thymic architecture rather than due to a cell autonomous defect [33]. DC development and function was found to be normal in mice lacking individual p50, RelA and cRel NF-κB subunits. However, development of DC was significantly impaired in double deficient $p50^{-/-}$ $RelA^{-/-}$ mice. In contrast, DC from $p50^{-/-}$ $cRel^{-/-}$ mice developed normally, but showed impaired survival of mature DC [34]. The loss of DC was also observed in the $p50^{-/-}$ $p52^{-/-}$ mice as these mice lack both dimerization partners for RelB [35].

Besides regulating DC development, NF-κB is also necessary for regulating the T cell stimulating function of DC since $RelB^{-/-}$ mice are impaired in antigen presenting [31, 32]. Interestingly, *in vitro* differentiated DC from $RelB^{-/-}$ mice showed a significant ability to stimulate T cells. However, they were not able to form aggregates with naive T cells [36]. Therefore, RelB might regulate the DC-T cell interaction rather than antigen presentation by DC.

4.4
PU.1

PU.1 is an Ets family transcription factor required for development of both myeloid and lymphoid cells. While PU.1 is important for several lineages, the precise role of PU.1 in determining whether a hematopoietic progenitor differentiates into a macrophage, granulocyte or B cell has remained unclear. Recent studies suggested that the cellular concentration of PU.1 directly affects cell fate, with the highest concentrations of PU.1 being required for macrophage development and lower concentrations for granulocytic and B cell fate adoption. PU.1 transactivation activity is inhibited by the granulocytic transcription factor C/EBP and the B cell transcription factor Pax5/BSAP and thus high concentrations of PU.1 might be required for macrophage development in order to overcome the negative effects of alternative lineage specific factors. PU.1 upregulation has also been implicated in the maturation of myeloid cells after commitment towards the macrophage and granulocytic lineages.

High levels of PU.1 induce several myeloid genes, including Mac-1 (CD11b), F4/80, GM-CSF receptor and M-CSF receptor (CD115) [37–41]. Lack of PU.1 leads to a block in macrophage development and impairs also the development of other myeloid cell types [42, 43]. However, PU.1 is also required for the generation of lymphoid lineages, albeit at lower levels. PU.1 knockout mice lack B cells and are

deficient in fetal thymocytes [43, 44]. At low levels – but not at high levels – PU.1 directly induces the receptor for the canonical lymphoid cytokine IL-7 [45, 46]. Low levels of PU.1 activity are suspected to promote lymphoid cell development through IL-7 dependent pathways and through the absence of competing myeloid and erythroid gene expression.

The regulation of some of these PU.1 dependent genes involves heterodimeric complexes of PU.1 with a variety of other transcription factors and co-activators including C/EBPs, HMG I/Y, NF-κB family members, IRF-4, and IRF-8/ICSBP [47–52]. In some instances, binding of both factors to DNA is required; in others, PU.1 may actually tether other factors to the DNA, as reported for C/EBPβ on the IL-1β promotor [53]. Primarily recognized as a transcriptional activator, there is also emerging evidence that PU.1 can exert a repressive function. Interestingly, PU.1 has been reported to repress expression of I-Aβ MHC class II and CD11c [54, 55] which represent hallmarks of mature DC.

DC, similar to macrophages, express PU.1 and depend upon PU.1 for their development *in vivo* [56, 57] and over-expression of either PU.1 or of its close relative Spi-B, increases the propensity for DC development [58, 59]. Lymphoid precursors retain their ability to differentiate into DC (or in some instances into macrophages) until the point where the T- and B-lymphoid specification pathways diverge [60–64]. In T-cell differentiation, PU.1 downregulation, shortly followed by Spi-B downregulation, coincides with the end of the period in which DC or macrophage differentiation can occur [44, 61, 65, 66]. In B cells, PU.1 and Spi-B continue to be expressed throughout maturation. This might underlie the susceptibility of some B-lineage cell types to converge into macrophage-like cells, even after immunoglobulin gene rearrangement [62–64].

4.5 C/EBPα

C/EBPα is a transcription factor that is essential for granulocyte development [67]. C/EBPα has recently been shown to inhibit PU.1-induced transcription from reporter genes regulated by a multimerized PU.1 binding site. This repression is due to binding between the leucine zipper domain of C/EBPα and the Ets domain of PU.1. Similarly to GATA-1 binding to PU.1, C/EBPα binding results in the dissociation of c-Jun, which represses PU.1 transactivation. C/EBPα was also shown to participate in the tissue-restricted and regulated expression of CD11c through functional interactions with PU.1 [68]. Additionally, C/EBPα over-expression had an effect on cell fate decisions. Retroviral transduction of $CD34^+$ hematopoietic progenitors with a C/EBPα retrovirus inhibits PU.1 induced DC development and favors development of granulocytes. Thus, in myeloid progenitors the critical ratio between PU.1 and C/EBPα might direct cell fate.

Tab. 4.1 DC phenotypes of transcription factor knockout mice. A panel of transcription factor knockout mice and their DC phenotype (pDC, CD8α⁺ and CD8α⁻ DC subsets, LC) are shown. Several of these transcription factors also affect other hematopoietic lineages as depicted.

	B220⁺ pDC	**CD11b⁺ DC**		**CD11b⁻ CD8α⁺ DC**	**skin epidermal LC**	**Refer-ences**	**Role in other hematopoietic lineages**
		CD8α⁻ CD4⁻ DC	**CD8α⁻ CD4⁺ DC**				
Ikaros DN⁻/⁻	– (B220) – (B220)	–	– – (CD4)	– –	n.d. +	16, 20	Profound deficiency in lymphoid development, increased number of megakaryocytes and erythrocyte/myeloid precursors, normal myeloid development, disrupted splenic architecture
Ikaros C⁻/⁻	– (B220)	–	–	↓	n.d.	20	Defects in T cell, B cell and NK cell development, disrupted splenic architecture
PU.1⁻/⁻	n.d. n.d.	– n.d.	– n.d.	+ (CD8α⁺ Dec205⁺) – (Dec205)	n.d. n.d.	56, 57	Profound deficiency in B cell and myeloid development
RelB⁻/⁻	n.d. n.d.	↓ to – ↑	 ↓	+ ↑	+ n.d.	33, 109	Myeloid hyperplasia, normal lymphoid development, disrupted thymic architecture
Id–2⁻/⁻	↑ +	↓ +	↑ +	↓ to – ↓ to –	– n.d.	99, 100	Deficiency in NK cell development

Tab. 4.1 Continued.

	B220$^+$ pDC	CD11b$^+$ DC		CD11b$^-$ CD8α$^+$ DC	skin epidermal LC	Refer-ences	Role in other hematopoietic lineages
		CD8α$^-$ CD4$^-$ DC	CD8α$^-$ CD4$^+$ DC				
IRF–8/ICSBP$^{-/-}$	↓ to –	+	↑	↓ to –	n.d.	88,	Defects in myeloid development
	↓ to –	n.d.	n.d.	n.d.	n.d.	92,	Defective DC maturation
	n.d.	n.d.	n.d.	n.d.	↓	93,	
	n.d.	+	+	–	n.d.	94,	
	↓	+	↑	↓ to –	n.d.	95	
IRF–4$^{-/-}$	+	+	↓	+	n.d.	87,	Impaired T and B cell function
	↓	+	↓	+	n.d.	88	
IRF–2$^{-/-}$	n.d.	n.d.	↓	↑	↓ CD4$^+$	85	Defects in B cell and NK cell development
Runx3$^{-/-}$	n.d.	↓		↑	–	105	Defects in T cell development
Gfi1$^{-/-}$	↓	↓	↓	↓	↑	106	Defects in T cell and neutrophil development

– = absent; + = present; ↑ = number of cells increased; ↓ = number of cells decreased; n.d. = not determined

4.6 Pax5

The transcription factor Pax5/BSAP represents a potential antagonist of PU.1 in development of myeloid *versus* lymphoid cells. Pax5/BSAP is a key transcription factor in B cell commitment and critical in suppressing alternative lineage cell fates early in B cell development [69]. In the absence of Pax5, B cell differentiation can only proceed to the pro-B cell stage. *In vitro* $Pax5^{-/-}$ pro-B cells mis-express myeloid target genes, such as the cytokine receptors M-CSFR and GM-CSFR, and make them acquired the potential for differentiating into both myeloid and T lineage cells [69, 70]. Several of the mis-expressed gene products are targets of PU.1, and Pax5 has been demonstrated to repress the transactivation activity of PU.1 [71]. Additionally, expression of Pax5 in myeloid cell lines and primary progenitors can repress the expression of several PU.1 target genes [72]. Thus, to induce myeloid differentiation of uncommitted cells PU.1 might have to overcome inhibitory actions of Pax5 and/or other B cell transcription factors.

$Pax5^{-/-}$ pro-B cells can differentiate into T cells, NK cells and myeloid cells including $CD11c^+$ $CD11b^+$ DC [69, 70]. Recent work using a Pax5 conditional knockout mouse has revealed that Pax5 expression must be maintained through the pro-B stage, as B-lineage committed pro-B cells revert to multilineage potential upon loss of Pax5 [73]. Pax5 also inhibits the expression of the GM-CSF receptor α-chain in cell lines, thereby potentially providing a mechanism for suppression of myeloid lineage cell fate [72].

4.7 IRF family

Transcription factors of the interferon (IFN) regulatory factor (IRF) family play a critical role in development and function of several immune cells (reviewed in [74–76]). IRFs were initially identified as regulators of IFN-α/β genes and to date nine members (IRF-1 to IRF-9) have been found in human and mouse. The expression of IRF-4 (Pip, LSIRF, ICSAT, MUM1) and IRF-8 (ICSBP) is restricted to hematopoietic cells, while the other IRFs are ubiquitously expressed.

IRFs share a conserved DNA binding domain in the amino terminal 115 amino acids (aa) that recognizes similar DNA target sequences. This region contains a characteristic conserved tryptophan repeat (five tryptophans spaced in 10–18 aa intervals), which is also found in the DNA binding domain of the Myb oncoproteins [74]. Despite their similar DNA binding specificity, studies characterizing IRF expressing cell lines and IRF knockout mice reveal that each member of the IRF family exerts a distinct role in biological processes such as pathogen responses, cytokine signaling, cell growth regulation and hematopoietic differentiation. To date gene targeting studies in mice for IRF members were performed for all IRFs except IRF-6.

Cellular resistance against virus is mediated by the IFN induced gene expression system. Stimulation of the type I IFN receptor (IFNAR) induces specific IRF family members to bind interferon stimulated response elements (ISRE), while type II IFN receptor (IFNGR) induces IRF binding to gamma-activated sequence (GAS) elements in IFN responsive genes [75, 77]. The essential role for IRF-3, -7 and -9 in activation of IFN-α/β genes in response to virus infection, was recently shown and has been extensively discussed in other reviews [75, 76]. However, they were not described to be involved in developmental processes within the hematopoietic system. IRF-5 and IRF-6 are structurally related to one another but information regarding their functions has been scarce. The expression of IRF-5 is induced by IFN-α/β stimulation suggesting its participation in the IFN system. Recently, Takaoka et al. [78] demonstrated that IRF-5 is generally involved downstream of the TLR-MyD88 signaling pathway for gene induction of pro-inflammatory cytokines, such as IL-6, IL-12 and TNF-α.

An important role for development of hematopoietic cells was shown for mice deficient for IRF-1, IRF-2, IRF-4 and IRF-8. IRF-1 transcription factor was originally identified as a regulator of virus-inducible enhancer-like elements of the human IFN-β gene [74, 75]. Moreover, mice deficient in the IRF-1 gene show reduced numbers of NK cells (NK1.1$^+$ TCRα/β^-), NK-T cells (NK1.1$^+$ TCRα/β^+) and γ/δ T cells [79-81]. However, IRF-1$^{-/-}$ bone marrow (BM) cells can generate functional NK cells when transplanted into irradiated WT mice indicating that IRF-1 selectively affects stroma cells that constitute the microenvironment for NK cell development but not for NK cell progenitors [80]. In contrast, while having normal numbers of immature CD8$^+$ and CD4$^+$ thymocytes, IRF-1$^{-/-}$ mice are 90% deficient in mature CD4$^-$ CD8$^+$ T cells in the thymus and impairment of CD8$^+$ T cell maturation is cell autonomous [82]. This is explained by defective control of positive and negative selection of CD8$^+$ thymocytes in the thymus. From such a mechanism one would not reason for a role for IRF-1 on the development of e.g. the lymphoid related CD8α^+ DC found in the thymus and there is no information for an altered DC phenotype in IRF-1$^{-/-}$ mice.

IRF-2 was identified due to its high homology to IRF-1 and was originally described as an antagonist of the IRF-1-mediated transcriptional regulation of IFN-inducible genes. From these initial observations it was suggested that IRF-1 and IRF-2 function as transcriptional activators and repressors, respectively [75, 77]. But IRF-2 was also shown to act as a transcriptional activator of genes, such as for VCAM-1 and gp91phox [83, 84]. IRF-2$^{-/-}$ mice were also shown to be defective in proper NK cell development but in contrast to IRF-1$^{-/-}$ this is due to a cell-autonomous mechanism that affects NK cell progenitors but not NK-T cells [85]. Most importantly, IRF-2$^{-/-}$ mice were found to exhibit a selective cell autonomous defect in splenic CD4$^+$ CD11b$^+$ DC [85]. Furthermore, the numbers of LC in IRF-2$^{-/-}$ mice were reduced at least in part due to the lack of the CD4$^+$ CD11b$^+$ LC subset. Interestingly, these deficiencies in DC were diminished in double knockout mice lacking both IRF-2 and the IFN-α/β receptor which indicates that IRF-2 acts through negatively regulating IFN-α/β signals. In contrast, NK cells still showed developmental arrest in these double mutant mice, suggesting that the mode of

action of IRF-2 for $CD4^+$ DC development is distinct from that for NK cell development [85].

IRF-4 and IRF-8 are predominantly expressed in lymphoid cells. IRF-4 is known to form a transcriptional complex together with PU.1 that is implicated in regulation of B cell–specific genes, such as the immunoglobulin light chain (IgL) genes or the CD20 gene [47–49]. PU.1-IRF-4 interaction requires PU.1 binding to DNA with subsequent recruitment of IRF-4 via a phosphorylated serine residue (Ser-148) in the PEST region of PU.1. It was found that IRF-4 can also form ternary complexes on an Ets-IRF composite element (EICE) with Spi-B in a manner similar to IRF-1-PU.1 [77]. IRF-4 was initially described to be expressed at all stages of B cell development, in mature T cells and in macrophages. Analysis in IRF-4 deficient mice revealed that IRF-4 is essential for the function and homeostasis of both mature B and mature T lymphocytes [86]. At 4 to 5 weeks of age, lymph nodes and spleens of $IRF4^{-/-}$ mice showed normal lymphocyte distribution but developed progressive generalized lymphadenopathy, because of an expansion of T (both $CD4^+$ and $CD8^+$) and B lymphocytes. IRF-4-deficient mice exhibited a profound reduction in serum immunoglobulin concentrations and did not mount detectable antibody responses. T lymphocyte function was also impaired *in vivo* and these mice did not generate cytotoxic or anti-tumor responses [86]. More recently IRF-4 was found to be expressed specifically in splenic $CD4^+$ $CD8\alpha^-$ $CD11b^+$ DC and in $IRF4^{-/-}$ mice this DC population was severely reduced [87, 88]. Although bone marrow transfer studies were not performed with $IRF\text{-}4^{-/-}$ mice, in gene transfer experiments IRF-4 transduction of $IRF\text{-}4^{-/-}$ cells rescued the DC deficiencies *in vitro* pointing to a cell-intrinsic mechanism [88].

Amongst all IRF family members, IRF-8 shares the highest degree of homology with IRF-4. Like IRF-2, IRF-8 was initially characterized as a repressor of genes with ISRE elements [89]. But IRF-8 has been also been described as a transcriptional activator. For example IRF-8 deficient mice lack expression of IL-12 p40 [90]. The promoter of the IL-12 p40 subunit contains both ISRE and GAS motifs. Similar to IRF-4, IRF-8 can form ternary transcriptional complexes on EICE in a PU.1- or Spi-B dependent manner. However, unlike IRF-4, the PU.1-IRF-8 complex is less active in transactivation due to the lack of an activation domain within IRF-8. The DNA binding activity of IRF-8 *per se* is very weak but dramatically increased by interaction with IRF-1 and IRF-2, and notably IRF-1 and IRF-2 knockout mice also display defective IL-12 expression [75, 77]. Beyond the immunodeficiency, $IRF\text{-}8^{-/-}$ mice develop a chronic myelogenous leukemia (CML)-like syndrome with a systemic expansion of granulocytes followed by a fatal blast crisis [90]. $IRF\text{-}8^{-/-}$ mice harbor an increased number of myeloid progenitor cells and the knockout progenitors preferentially give rise to granulocytes while macrophage development is impaired [90]. Studies with myeloid progenitors have shown that IRF-8 drives differentiation towards macrophages while concomitantly inhibiting granulocyte differentiation [91].

More recent studies demonstrated that IRF-8 is essential for development of $CD8\alpha^+$ DC, plasmacytoid DC (pDC) and epidermal LC [88, 92–95]. These studies describe the subset-specific expression of IRF-8 within the $CD8\alpha^+$ DC, pDC and

LC population and lack of these cells in IRF-8$^{-/-}$ mice. Not surprising, mice deficient in both IRF-4 and IRF-8 lack all conventional DC subsets and also pDC [88]. Interestingly, the double knockout of IRF-4 and IRF-8 resulted in a complete block in B cell development at the pre-B cell stage while the single loss of either IRF-4 or IRF-8 did not [96]. Studies with radiation bone marrow chimeras demonstrated that IRF-8 function in CD8α^+ DC development is cell-autonomous [92]. In *in vitro* gene transfer experiments IRF-8 expression in IRF-8$^{-/-}$ cells rescued also PDC development indicating a cell-intrinsic mechanism also for this DC subpopulation [88, 95].

4.8 Id2

Id2 is a member of the Id (inhibitor of DNA binding/differentiation) transcription factor family [97]. Id proteins belong to the class of basic helix-loop-helix (HLH) transcription factors and such factors have been demonstrated to play a pivotal role in lineage choice and differentiation [98]. Most HLH proteins, such as E2A, act as transcriptional activators and are antagonized by yet another class of HLH proteins referred to as Id proteins. Four Id proteins (Id1-Id4) have been identified so far [97]. Id proteins contain the highly conserved HLH domain but lack the basic regions needed for DNA binding. Thus, heterodimerization of Id proteins with HLH activators such as E2A leads to a complex that is incapable of DNA binding. Therefore, one activity of Id proteins is to act as dominant negative antagonists that titrate out the activity of the activating HLH transcription factors. As a result the picture emerges where Id proteins act as "buffers" that can sequester E proteins before they are released and engaged in specific developmental programs.

Transcriptional profiling of DC by DNA microarrays identified Id2 as a determining transcription factor of DC development [99, 100]. Id2 is upregulated during DC development in *in vitro* culture systems that recapitulate DC differentiation *in vitro*. Id2$^{-/-}$ mice lack LC, the cutaneous contingent of DC, demonstrating that Id2 is required for DC development *in vivo*. Id2$^{-/-}$ mice also lack CD8α^+ DC in spleen while the frequency of CD11c$^+$CD11b$^+$ myeloid DC was apparently not affected. TGFβ1$^{-/-}$ mice also lack LC and it was demonstrated that TGFβ1 induces Id2 transcription, and this places TGFβ1 signaling upstream of Id2.

The proportion of pDC was found to be higher in Id2$^{-/-}$ mice than in wild type control [99], as was the frequency of B cells [101]. Additionally, Id2$^{-/-}$ mice are in a Th2 dominant state and are also impaired in NK cell development [100, 102].

Thus, Id2 appears to have a selective impact on the development of various hematopoietic lineages including specific DC subsets. Therefore, a picture emerges in which the relative abundance of Id2 and e.g. E2A determines lineage choice by affecting the propensity of a common precursor to develop into B cells, NK cells and specific DC subsets: low or no expression of Id2 and high expression of E2A supports B cell and pDC development whereas high expression of Id2 blunts E2A activity and B cell and pDC development, and thus allow differentiation into NK cells and DC.

The other conditions that divide up the developmental space of lymphoid precursors are all permissive for at least some classes of DC. Notch signaling is not required for DC development but is also not inhibitory [103, 104]. In fact, Notch signaling can induce DC-like differentiation from monocytes while blocking their differentiation into macrophages. A variety of Id:E protein ratios are also permissive for DC in the broadest sense, although particular subsets are sensitive to high or low ratios [105]. This broad tolerance for Notch/bHLH conditions suggests that DC (and sometimes macrophage) development can result from an increase in PU.1 (or possibly Spi-B) activity across the whole plane of lymphoid-permissive conditions.

4.9 Runx3

Mature DC express the transcription factor Runx3 [106] that is a mediator of TGFβ signaling. Members of the runt domain family of transcription factors are key regulators of lineage fate decision in mammals. Runx proteins bind to specific target sequences and activate or repress transcription through recruitment of other transcription modulators. Runx3 is the smallest and founding member of this gene family and DC of Runx3$^{-/-}$ mice are insensitive to TGFβ induced maturation inhibition [106]. Additionally, Runx3$^{-/-}$ mice lack epidermal LC while the frequency of alveolar DC is increased. Runx3$^{-/-}$ DC also display an over-response to maturation stimuli which leads to an increased potency of Runx3$^{-/-}$ DC for T cell stimulation. These findings relate well to the finding that Runx3$^{-/-}$ mice develop spontaneous eosinophilic lung inflammation due to an over-response to otherwise harmless airborne antigens, which is probably due to a lack of responsiveness to locally secreted TGFβ. Thus, Runx3 appears to have a function both in regulation of DC development and DC activity.

4.10 Gfi1

Gfi1 (growth factor induced 1) represents a nuclear zinc finger protein that functions as a transcriptional repressor. Initial studies demonstrated a function of Gfi1 in hematopoietic stem cell maintenance, T cell and granulocyte development and most recently also in DC development and function [107 and references therein]. Hematopoietic stem cells, DC precursor cells and DC express Gfi1 and its expression is induced by GM-CSF, a cytokine that is important for DC development *in vitro*. Most importantly, Gfi1$^{-/-}$ mice showed a global reduction of myeloid and lymphoid DC subsets whereas LC frequencies were enhanced [107]. Gfi1 appears to be also important for DC function since Gfi1$^{-/-}$ DC were defective in maturation and exhibited an increased cytokine production.

Since Gfi1$^{-/-}$ progenitors from bone marrow failed to develop into DC *in vitro* but differentiated into macrophages, suggests that Gfi1 is a critical modulator of

DC *versus* macrophage development. Most significantly, the developmental deficiencies of Gfi1$^{-/-}$ progenitors were associated with decreased STAT3 activation. Given the fact that STAT3 is a critical component of GM-CSF receptor signaling and that STAT3$^{-/-}$ mice also exhibit deficiencies in DC development [108] further emphasizes the impact of the Gfi1-STAT3 axis in DC.

Many studies have demonstrated the impact of the Jak-Stat pathway in cytokine receptor signaling, including the Flt3 receptor. Given the importance of Flt3 ligand (Flt3L) on DC development (see Chapter 2), STAT3 signaling in DC was analyzed in detail [108]. STAT3$^{-/-}$ mice exhibit a profound atrophy in the DC compartment and lack both CD8α^+ and CD8α^- DC in spleen and lymph nodes. Importantly, STAT3 deficiency abrogated Flt3L specific effects on DC development and normal DC numbers could not be restored by Flt3L treatment. These findings indicating that STAT3 is an essential component in Flt3L induced DC development *in vivo*.

4.11 Concluding Remarks

Over the past several years we experienced an increasingly detailed understanding of the identity and mechanisms by which transcription factors determine commitment and differentiation of hematopoietic cells, including DC. DC develop from HSC and multipotent progenitor cells that exhibit a broad gene expression repertoire and express genes of several lineages but at low levels. During hematopoiesis the gene expression repertoire of such cells becomes increasingly restricted thereby leading to the establishment to distinct developmental lineages from the choice of several.

Hematopoiesis is generally viewed as a hierarchical and cell-autonomous process that depends on orchestrated gene expression programs driven by specific transcriptional regulators. Stem/progenitor cell renewal and cell fate choice in hematopoiesis have been proposed to be regulated by instructive signals provided by the microenvironment including cell-to-cell contacts, cytokines, Wnt and Notch factors. However, all extrinsically activated signaling pathways within stem/progenitor cells will amalgamate in modulating the gene activity of lineage determining transcription factors.

Therefore, powerful techniques such as fluorescence activated cell sorting and analysis, gene expression profiling and gene targeting strategies with knockout, knockin and transgenic mice have provided a more complete understanding of the transcriptional regulators that delineate DC development. Targets of these regulators have been identified, thereby providing first clues on the molecules that direct DC differentiation and on their impact for inhibiting alternative cell fates. Certainly, to fully understand transcriptional control of DC development will require the identification of all critical gene targets of these transcription factors and how their expression is regulated.

The search of further transcriptional regulators and the identification of novel DC associated and/or specific transcription factors are expected to shed light into the current controversy of the developmental origin of DC and their subsets.

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II
Sentinel Dendritic Cells in Nonlymphoid Organs

5
Epidermal Langerhans Cells

Nikolaus Romani, Christoph H. Tripp, Gudrun Ratzinger, Christine Heufler, Franz Koch, Sem Saeland and Patrizia Stoitzner

5.1
Introduction and Definition

Langerhans cells are defined as those dendritic cells that reside in the epidermis and in pluristratified epithelia. Their most selective (with some restrictions) molecular marker is the langerin/CD207 molecule [1, 2] (Fig. 5.1); their unequivocal ultrastructural marker is the rod- or tennis racket-shaped Birbeck granule [3, 4] (Fig. 5.2). Langerhans cells have long served as a useful paradigm for dendritic cell function in general [5]. This paradigm is currently shifting as it becomes increasingly clear that Langerhans cells are not only a local variant of dendritic cells, but rather, that they may possess some typical properties of their own [6]. This development can be "re-enacted" in a series of Langerhans cell-specific review articles and books that were written over the past years by Wolff 1972 [7], Silberberg-Sinakin et al. 1980 [8], Rowden 1981 [9], Wolff and Stingl 1983 [10], Schuler 1991 [11], Moll 1995 [12], Maurer and Stingl 2001 [13], Romani et al. 2003 [6], and finally Wilson and Villadangos 2004 [14], and that are still worth reading today.

5.2
A Short Review of the History of Langerhans Cells

In 1868 the medical student Paul Langerhans described dendritically shaped cells in the human epidermis [15]. He found them by means of a then state-of-the-art gold chloride impregnation technique for the identification of nerve cells. The use of this method as well as his observation that some of the dendrites appeared to extend down into the dermis led to the conclusion that these cells were some sort of

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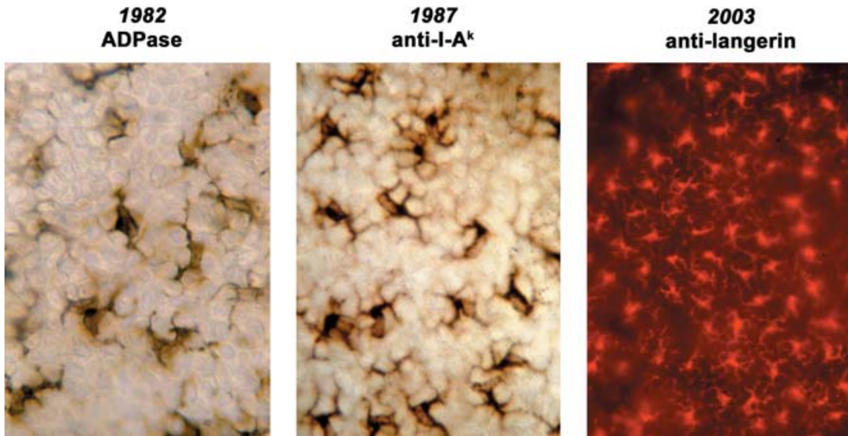

Fig. 5.1 Langerhans cells in epidermal sheet preparations, as visualized over the years. One of the first molecules that served as a signature for Langerhans cells *in situ* was ATPase or ADPase. A photo from 1982 shows the immunocytochemical visualization of Langerhans cells by means of ADPase activity. As monoclonal antibodies became available, anti-Ia (i.e. anti-MHC class II) antibodies were widely used to detect Langerhans cells in the epidermis. This is depicted in the photo from 1987 using an immunoperoxidase detection technique. More recently, the langerin/CD207 molecule was recognized as the most selective Langerhans cell marker. The picture from 2003 shows mouse Langerhans cells *in situ*, detected by monoclonal anti-langerin antibody using an immunofluorescence technique. Note that the magnifications are not equal.

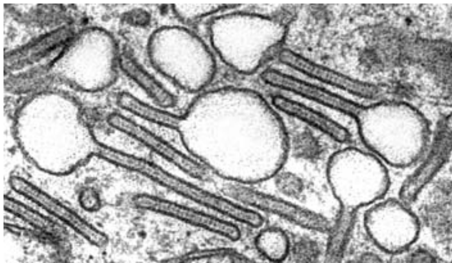

Fig. 5.2 Langerhans cells by electron-microscopy: Birbeck granules. In this high power picture of a human Langerhans cell, Birbeck granules can be readily seen by the conspicuously parallel membranes that give them the typical rod- and tennis-racket-like shapes. Thickness of the rod portion is approximately 50 μm [4].

nerve cell. Langerhans cells kept this label for more than a century. The advent of electronmicroscopy would change the views. For a while, Langerhans cells were considered as effete, i.e. "worn out" melanocytes. The discovery of Birbeck granules both in clear cells (i.e. Langerhans cells) of vitiliginous epidermis [3] as well as in cellular infiltrates of the so-called "histiocytosis X" [16] established the first link between Langerhans cells and the immune system. The second piece of strong evidence that Langerhans cells might belong to the hematopoietic system in general and to the mononuclear phagocyte family in particular was provided by the demonstration of surface ATPase on Langerhans cells [17]. Finally, three sets of landmark studies made it clear where Langerhans cells belong to ontogenetically. *First*,

the demonstration of "immune molecules" like Ia molecules, complement- and Fc-receptors was compelling evidence of a leukocyte nature of Langerhans cells [18–20]. *Secondly*, chimera studies provided the final proof for the bone marrow-derivation of Langerhans cells [21, 22]. And *thirdly*, the seminal work of Schuler and Steinman gave Langerhans cells their final place as a member of the dendritic cell system [5, 23]. An excellent account of the history of Langerhans cells was written by Klaus Wolff, one of the founder-fathers of modern Langerhans cell research [24]. A rich source for historical facts, documents and pictures of Paul Langerhans is a book by Björn Hausen entitled "Die Inseln des Paul Langerhans" ("The islets of Paul Langerhans") [25].

5.3 Characterization and Morphology of Langerhans Cells

When one characterizes Langerhans cells one normally refers to Langerhans cells *in situ* in the epidermis or Langerhans cells isolated from the epidermis. One should, however, be aware of the fact that we can now also find, define and describe those Langerhans cells that have migrated from the epidermis to the draining lymph nodes [14]. Moreover, several experimental methods are now available to obtain Langerhans cells or rather, Langerhans cell-like cells by culture from monocytes [26] or from hematopoietic stem cells [27, 28]. Therefore, it is important to critically differentiate between these types of Langerhans cells whenever reading or discussing Langerhans cell phenotype or function.

The dendritic shape of Langerhans cells *in situ* is principally not different to that of other dendrite-forming cells of the epidermis such as melanocytes or dendritic epidermal T cells. The network-like appearance of Langerhans cells (Fig. 5.1) has occasionally provoked the question whether these cells might form a contiguous, interconnected cellular net. Contacts between individual Langerhans cells could never be found, however. The above-mentioned Birbeck granules are a useful marker for Langerhans cells, at least for those *in situ* and in an immature state. Upon maturation of Langerhans cells the number of these granules is downregulated; sometimes, e.g. after a 3-day culture in the murine system, they even disappear [5]. A common observation of Langerhans cell researchers was that Birbeck granules are less abundant in mouse than in human Langerhans cells. Langerhans cells that have arrived in the lymph nodes in response to an antigenic challenge appear to still possess at least some Birbeck granules [29] even though this issue has not yet been systematically studied. This question will become more amenable to study now that models are being devised where Langerhans cells can be tagged with fluorescent proteins expressed under control of the langerin promoter and will thus be more easily retrievable (and "sortable") from the lymph nodes [30, 31].

One terminology-related remark may be permitted at this point. The term "dendrite" describes the long, often branched, cellular processes that contain a normal set of cell organelles like mitochondria etc. Langerhans cells *in situ* possess dendrites. When Langerhans cells (or other types of dendritic cells) are placed in cul-

ture and allowed to mature they form what researchers often call “veils” [32, 33]. These long, thin and motile cellular processes are largely devoid of organelles except ribosomes. *In vivo*, “veils” can be observed in dendritic cells floating in lymph vessels [34–36].

5.4
Entry of Langerhans Cells into the Epidermis in Ontogeny and Adulthood

The above-mentioned experiments with chimeric mice proved that Langerhans cells were derived from the bone marrow [21, 22]. Similarly, this could be shown in bone-marrow-transplanted human individuals [37, 38] using the male Y-bodies as a cytogenetic marker for male donor Langerhans cells in female recipient epidermis. Whereas the general derivation of Langerhans cells thus appears clear, the question as to how exactly Langerhans cell precursors populate the epidermis both in ontogeny, i.e. in the fetal and perinatal period, and under homeostatic conditions is still under investigation.

5.4.1
Entry of Langerhans Cells into the Epidermis during Ontogeny

Langerhans cell precursors populate the epidermis during fetal and early postnatal life. In the mouse a network of Langerhans cells comparable to the adult is reached only about two weeks after birth. Three key features of ontogenetic development were identified, mainly in mouse models. *First*, Langerhans cells entering the epidermis acquire their molecular outfit in a stepwise fashion. The first markers, by which epidermal leukocytes can be recognized, are CD45 and ATPase or ADPase. Only shortly before birth do Langerhans cells acquire expression of MHC II molecules [39, 40]. Even later, they display C-type lectin receptors such as langerin/CD207 [41] and DEC-205/CD205 [42]. It is obvious that this has implications with regard to the functional repertoire of Langerhans cells in the early postnatal period [42, 43]. In a less comprehensive way – understandably – this has also been studied in human skin [44]. *Second*, it appears that IL-10 may be a critical cytokine within the perinatal epidermal milieu in that it restrains differentiation and maturation of Langerhans cells and is therefore perhaps responsible for the stepwise acquisition of immune molecules by developing Langerhans cells [45]. *Third*, recent work has shed light on the issue whether all Langerhans cells enter the epidermis as precursors, or whether a few Langerhans cell precursors enter, and multiply and differentiate further within the epidermis. Chang-Rodriguez et al. have observed that in newborn and early postnatal epidermis a large percentage of MHC II$^+$ cells, i.e. Langerhans cells, are actively dividing. *In vitro* experiments showed that these cells, when isolated and enriched, have the potential to acquire Langerhans cell characteristics such as expression of langerin and DEC-205 [46]. Most likely, they also do so *in vivo*, as suggested by experiments where fetal skin was grafted onto adult mice, and the Langerhans cell precursor of the graft multiplied and differentiated [46].

Little is known about the regulation of Langerhans cell entry into the epidermis during perinatal life by cytokines and chemokines. With one notable exception (see below), essentially all cytokine- or chemokine deficient mice as well as cytokine/chemokine receptor knock-out mice studied thus far have not shown any gross alterations in their Langerhans cell densities. Of course, this applies only to those relatively few studies where Langerhans cells were specifically addressed. Even mice that do not express CCR6, the receptor for CCL20, have normal Langerhans cells ([47], and our own, unpublished observations). CCL20/macrophage inflammatory protein 3-α/MIP3-α is the important chemokine that selectively attracts Langerhans cell precursors into the epidermis, and that is produced by epithelial cells [48–50], albeit predominantly under inflammatory conditions.

The above-mentioned exception is the cytokine TGF-β1 and the TGF-β1-induced transcription factor Id2. Mice that are deficient in this cytokine or transcription factor have a dramatic phenotype in that they totally lack Langerhans cells in the epidermis [51–53]. TGF-β production by keratinocytes seems to be critical for the entry of Langerhans cell precursors into the epidermis. The lack of TGF-β-induced Id2 in Langerhans cells (which may be assumed from the report by Zenke's group [53] but was not directly shown) has no consequences. This may be concluded from experiments where bone marrow cells from TGF-β1-deficient mice gave rise to Langerhans cells when injected into normal TGF-β-competent mice [52]. There may be additional members of the TGF family of cytokines that also have an influence on Langerhans cell homing to the epidermis. The neutralization of Activin A, one such molecule, resulted in a marked reduction, though not total absence, of Langerhans cells in the epidermis [54]. The precise mechanism of action of TGF-β and Id2 has not been elucidated yet.

5.4.2
Entry and Turnover of Langerhans Cells into the Adult Epidermis under Homeostatic Conditions

It has been known since the original, above-mentioned bone-marrow transplantation experiments that Langerhans cells can persist and survive within the epidermis for a long time, much longer that all the other recipient leukocytes. In a xenogeneic skin transplantation setting Krueger et al. showed that graft Langerhans cells persisted in the skin transplants for many weeks [55]. Merad et al. [56] carried these observations even further. They reconstituted lethally irradiated mice with T cell-depleted bone marrow. This caused no graft-versus-host disease. Under these noninflammatory circumstances host Langerhans cells stayed in the epidermis for more than a year. Furthermore, Kanikatis et al. have recently shown in the first double human hand allograft that epidermal Langerhans cells remarkably remained of donor origin over the 4.5 year follow-up period studied [57].

These findings can be attributed to an exceptional longevity and to a very low rate of Langerhans cell division. Evidence showing the potential of Langerhans cells *in situ* to divide has been corroborated over the years. Mitoses were spotted in the light and electron microscope in normal, unirradiated epidermis [58, 59]; BrdU or

tritiated thymidine incorporation into Langerhans cells *in situ* [56, 60, 61] was observed; and flow cytometric DNA measurements also indicated Langerhans cell division [62]. Longevity was recently underscored by the virtual absence of apoptosis in Langerhans cells in untreated steady state epidermis [63].

Where do these few dividing Langerhans cells come from? Merad's observations [56] would suggest that they do not stem from blood precursors in the steady state. Rather, the skin itself, i.e. epidermis or dermis, may harbor a Langerhans cell precursor that is responsible for homeostasis. A CD14+/CD207-langerin+ dermal cell has been discussed in that regard [64]. Recent observations render this possibility less likely. It was observed, that both in fetal epidermis [41, 46] and in an experimental system where bone marrow progenitors were injected into the skin [65] the newly arriving cells in the epidermis did not yet express the langerin molecule. Alternatively, the epidermis itself may harbor the precursor for Langerhans cells. In the adult epidermis the proportion of actively cycling epidermal leukocytes is about 2–5% of all MHC II-expressing cells. This was determined by the detection of cyclin B1, a protein that is specifically expressed during mitosis [46]. Thus, this assay reflects only ongoing cell division rather than also detecting past division events, as the BrdU incorporation assays would do. Therefore, it seems that the proliferating precursor indeed resides within the epidermis rather than stemming from the dermis, where it divides, picks up the BrdU, and then only enters the epidermis. Together with the few but unequivocal ultrastructural observations of mitotic Langerhans cells, defined as such by the presence of Birbeck granules, in normal uninflamed epidermis [59, 66, 67] this evidence would strongly suggest that homeostatic proliferation takes place within the epidermis proper.

It was not directly assessed whether these proliferating epidermal Langerhans cell precursors express also langerin. Even though it was repeatedly observed that in the adult epidermis there is virtually a complete overlap of MHC II and langerin expression [2, 68], such a minute population may have been overlooked in that regard. Thus, the question whether the dividing cells within the epidermis are phenotypically fully differentiated Langerhans cells (i.e. langerin$^+$) or still display phenotypical features of a precursor cells (e.g. langerin$^-$ cells) cannot definitively be answered. Again, the ultrastructural demonstration of Birbeck granules, that consist of langerin molecules to a substantial part [1], in mitotic cells would suggest that it is fully differentiated Langerhans cells that proliferate.

5.4.3 Entry and Turnover of Langerhans Cells into the Adult Epidermis under Inflammatory Conditions

Langerhans cell repopulation of the epidermis during inflammation is different from the homeostatic situation. It occurs at a much faster pace. When the epidermis is depleted of Langerhans cells to varying degrees by inflammation-inducing mechanical (e.g. tape stripping [65]) or physico-chemical treatments (e.g. UV light [56], corticosteroids [69]) it takes only few weeks until the original density of Langerhans

cells in the epidermis is re-established by repopulation with precursors that originate in the bone marrow and enter the skin via the blood.

As in ontogeny, Langerhans cell precursors that enter the epidermis in response to an inflammatory stimulus do not yet express langerin but express substantial levels of CCR6. This was observed in a murine tape-stripping model [65]. The most likely Langerhans cell precursor was described in human blood as a $CD1a^+$/$CD11c^+$ subset of blood dendritic cells. When these cells were cultured in the presence of TGF-β they quickly gave rise to cells with all the characteristics of Langerhans cells, i.e. expression of langerin, E-cadherin and Birbeck granules [70]. An *in vivo* observation of several years ago seems to corroborate this notion: an increased percentage of $CD1a^+$ circulating cells was found in the blood of patients with burns, i.e. with extensive inflammation in the skin [71]. Under the aegis of TGF-β1 it is possible *in vitro* to obtain Langerhans-like cells even from monocytes [26]. Whether this pathway contributes to the replenishment of Langerhans cells in inflammatory situations is not known.

Unlike in ontogeny, the chemokine ligands for CCR2 (CCL2/monocyte chemoattractant protein-1/MCP-1, CCL7/MCP-3) and CCR6 (CCL20/MIP-3α) play critical roles in the repopulation of inflamed epidermis. Blood and bone-marrow precursor cells as well as Langerhans cells express CCR2 [56] and CCR6 [48, 65]. The respective chemokine ligands are expressed in epidermal keratinocytes both in mice [56, 72] and in humans [48, 49]. Importantly, CCL20 was found in the epidermis in various inflammatory dermatoses such as psoriasis [73], contact dermatitis, mycosis fungoides [50] and also in inflammatory bowel disease [74]. Accordingly, it was shown *in vivo* that precursor cells in the bone marrow that lack either CCR2 [56, 72] or CCR6 [72] have greatly reduced or lost their potential to give rise to epidermal Langerhans cells when injected into lethally irradiated recipient mice. Thus, MCP-1, MCP-3 and MIP-3α appear to be (sequentially active [75]) key regulators of Langerhans cell influx into the epidermis under inflammatory conditions, which in "real life" would often mean when microbial pathogens encounter the epidermis.

5.5 Lineage of Langerhans Cells

Dendritic cells can belong to two hematopoietic lineages, the myeloid or the lymphoid lineage. This distinction has recently become less rigid because dendritic cells of both lineages exert some functions that had originally been thought specific for one or the other lineage. The production of type I interferon is such an example. It can be secreted both by plasmacytoid dendritic cells, that are mainly lymphoid-derived [76], and also by myeloid dendritic cells [77]. Moreover, *in vitro* and in bone-marrow reconstitution experiments both myeloid-committed stem cells and lymphoid-committed stem cells can give rise to dendritic cells [78]. This is discussed in detail in Chapter 2 by Manz.

Also Langerhans cells can be found after injection of lymphoid-committed stem cells [79] and presumably also of purified myeloid-committed stem cells. Langerhans cells *in situ* appear to be of myeloid origin since they express typical myeloid marker molecules such as CD33 and CD11b. In accordance with Manz (Chapter 2) we would like to stress that both phenotypical markers as well as bone-marrow reconstitution experiments must be viewed with a grain of salt. Phenotypical features may change, and the fact that experimental injection of one or the other type of hematopoietic stem cell gives rise to Langerhans cells may not necessarily mean that this is so in "real life".

Additional evidence for a myeloid derivation of Langerhans cells stems from observations in mice that lack a critical transcription factor (IRF-8/ICSBP) for the myeloid lineage. The development of Langerhans cells is disturbed in such mice as evidenced by markedly lower densities of these cells in the epidermis [80]. Similarly, in mice lacking IRF-2 Langerhans cells are partly reduced [81], also emphasizing their myeloid derivation. Another set of transcription factors (RelB, Ikaros, PU.1, Notch-1) was found critical for the development of lymphoid cells. In addition, the absence of these factors in knock-out mice also led to a defect in dendritic cell development, mainly of the CD8-negative subset in lymphoid organs (except for Notch-1-deficient mice, who possess normal dendritic cells). Langerhans cells, however, were normal in these mice (RelB$^{-/-}$ [82], Ikaros$^{-/-}$ [83], Notch-1$^{-/-}$ [84]. This would argue against a lymphoid derivation of Langerhans cell *in vivo*. Inversely, when CD34+ stem cell were transduced with the myeloid lineage-favoring factor PU.1 an enhanced generation of Langerhans cells in culture was noticed [85]. In Chapter 4, Zenke discuss in more detail the roles of these and other transcription factors for the development of different subsets of dendritic cells. Taken together, Langerhans cells under homeostatic conditions are most probably of myeloid derivation.

5.6 Langerhans Cells in Lymphoid Organs

Typically, Langerhans cells migrate from the epidermis to draining lymphoid organs (see below). For years researchers wished to find and characterize these Langerhans cells there. The ease of identifying Langerhans cells in the epidermis contrasted with the inability to unequivocally define them in lymph nodes. In humans this was not feasible because it is essentially impossible to obtain healthy lymph nodes (except tonsils). Another main obstacle in humans, but even more so in mice, was the absence of markers for Langerhans cells. Even though the physical presence of Birbeck granules was occasionally used as an ultrastructural marker [29], this approach did not allow extensive phenotypical analysis, let alone functional studies. Definition of epidermis-derived Langerhans cells in the lymph nodes by their expression of E-cadherin [86] or an epithelial antigen, gp40 met with some success[51, 87, 88]. Also the use of distinct expression patterns allowed the Langerhans cell population in the lymph nodes to be narrowed down. Ruedl et al.

defined Langerhans cells as CD11c^{high}/CD40^{high} [89]; Henri et al. delineated an MHCII^{high}/DEC-205^{high}/CD40^{high}/CD8α^{-} population as the putative skin-derived Langerhans cells [90]. A breakthrough came when Sem Saeland's group characterized an indispensable molecular cornerstone of the Birbeck granule, the C-type lectin langerin/CD207 [1, 2, 91, 92]. Antibodies against langerin became available and for the first time it was possible to track migrating Langerhans cells [68] and to characterize them in the lymphoid organs [93–95].

Some main features emerged in a number of recent studies that used anti-langerin reagents to specifically investigate Langerhans cells. Firstly, it became obvious that langerin^{+} cells occurred in large numbers only in lymphoid organs that drain the skin [68, 93] or the mucous membranes, such as the tonsil [91]. Mesenteric lymph nodes, for instance, have only few langerin^{+} cells [68]. The spleen is an exception in that regard. The CD8α^{+} fraction of spleen dendritic cells was found to express langerin [96]. This did not correlate with the presence of Birbeck granules, though [93]. Therefore, it remains unclear whether this langerin expression reflects any developmental or functional link between Langerhans cells and CD8α^{+} spleen dendritic cells. Secondly, Langerhans cells in the lymph nodes localize to the T-cell areas, as expected [2, 68]. Thirdly, langerin^{+} cells in the lymph nodes of BALB/c mice segregate into two subsets based on their CD8α expression. The majority do not express CD8α; they appear phenotypically mature as judged by their surface levels of CD40, CD80, CD86 [93] and the intracellular expression of 2A1 [68], a molecule typical for mature murine dendritic cells [97]. A smaller subset of CD8α^{+} lymph node also expresses langerin but displays an immature phenotype. It should here be emphasized that the size of the CD8α^{+}/langerin^{+} subset is strain-dependent, as it is virtually absent from lymph nodes and spleen of C57BL/6 mice [98]. Some pieces of evidence allow the conclusion that the small CD8α^{+} subset stems from the blood, but the major CD8α^{-} fraction of langerin-expressing cells in lymph nodes indeed represents migratory Langerhans cells that have come from the epidermis. Thus, (a) when the fluorescent tracer FITC is "painted" onto the skin it can only be found on CD8α^{-} Langerhans cells in the lymph node [93]; (b) the CD8α^{-} subset is much smaller if not absent in lymphoid organs that do not get influx from the skin such as mesenteric nodes, spleen or thymus [93]; (c) using DEC-205 as a marker for a skin origin it also became evident that migratory Langerhans cells (and also migratory dermal dendritic cells) were negative for CD8α [99]. Fourthly, the anti-langerin reagents allowed, for the first time, specific study of phenotype and cytokine production by Langerhans cells in the lymph nodes in the steady state compared with an inflammatory situation. Expression of co-stimulatory molecules as well as IL-12 p40 production was increased in Langerhans cells from lymph nodes draining inflamed skin [100]. Future studies will more specifically address Langerhans cells in steady-state lymph nodes in an attempt to identify tolerance-inducing or -maintaining Langerhans cells. Fifthly, Langerhans cells in human lymphoid organs are less well studied than in the mouse. A seminal study by Geissmann et al. [94] suggests that most langerin^{+} cells in human lymph nodes are immature, as defined by the absence of DC-LAMP/CD208 expression. The authors investigated lymph nodes from patients with chronic dermopathic

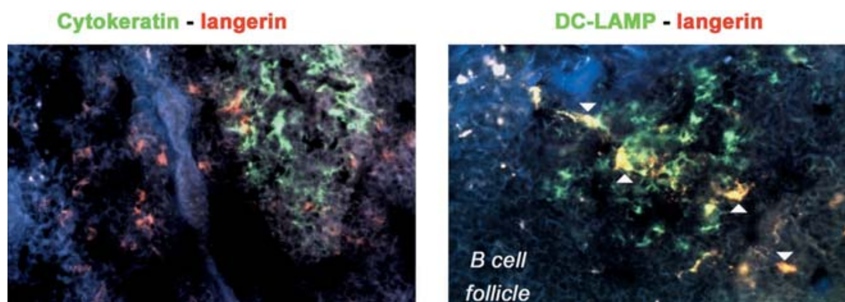

Fig. 5.3 Langerhans cells in the human tonsil. Cryosections of human tonsils were immunolabeled with monoclonal antibody DC-GM4 against human Langerin (red fluorescence). Left: the green fluorescence of double-labeling with antibody against cytokeratin shows that few Langerhans cells (red cells) are located within the epithelium of the tonsillar crypt, most Langerhans cells being found in the T-cell area. Right: in the T-cell area virtually all Langerhans cells express the maturation marker DC-LAMP (i.e. yellow/ orange fluorescence). Many mature non-Langerhans cells are present (green fluorescence of DC-LAMP^{+}/langerin^{-} cells). Immature Langerhans cells (red fluorescence of DC-LAMP^{-}/langerin^{+} cells) cannot be spotted in the T-cell zone of the tonsil.

lymphadenitis. This may not necessarily reflect the situation in a healthy lymph node. Surprisingly, there are no systematic studies yet about the maturational status of langerin^{+} cells in human tonsils. Our own unpublished observations indicate that langerin^{+} cells in the squamous epithelium are immature (just like in the epidermis). Those in the crypt epithelium express DC-LAMP to a large extent, whereas the vast majority of langerin^{+} cells in the interfollicular T-cell areas are DC-LAMP^{+}, indicating at least some degree of maturity [101], similar to the situation in the mouse (Fig. 5.3).

5.7 Langerhans Cells as a Paradigm for Dendritic Cell Function

Spleen dendritic cells were first described and characterized by Steinman and Cohn in the early 1970s [102]. At that time many *in vitro* studies used highly enriched populations of mature dendritic cells, i.e. after overnight culture of low density fractions of spleen cells; highly enriched populations of the immature stages that would correspond to dendritic cells *in situ* were not yet readily accessible. Isolation and short-term culture of epidermal Langerhans cells provided the first evidence for the profound morphological, phenotypical and functional changes that occur when dendritic cells mature [5, 103]. Dendritically shaped (*in situ*) or round (freshly isolated) cells changed to cells with pronounced cytoplasmic “veils”, MHCII molecules were upregulated, molecules typical for macrophages (F4/80, Fc receptor CD32) were downregulated, Birbeck granules disappeared, and the potent capacity to sensitize resting T cells was acquired. A little later it was shown that

the ability to process protein antigens for the MHC class II pathway developed in a reciprocal fashion. Both important uptake mechanisms [104] and processing capacity were shut down upon Langerhans cell maturation [105]. Early molecular and ultrastructural data explaining features of dendritic cell maturation were corroborated in the Langerhans cell system [106–108]. GM-CSF and TNF-α as essential maturation and survival factors for dendritic cells were first described using Langerhans cells [109–111]. Several of these findings were soon after confirmed with human Langerhans cells [112, 113]. For the more refined elucidation of molecular events during maturation, the Langerhans cell model proved highly useful in the recent past. It could clearly be demonstrated that MHCII molecules in Langerhans cell *in situ* were translocated from their intracellular pools to the cell surface [114] (Fig. 5.4). Finally, much of what we know about the migration of dendritic cells comes from studies on Langerhans cells or of skin dendritic cells in general that include Langerhans cells [115–119].

Thus, Langerhans cells and their properties were often viewed as being representative for all other types of dendritic cells. This is best exemplified in the wording of the title of the classical study by Schuler and Steinman that laid the foundations for the "Langerhans cell paradigm" [5]: "*Murine epidermal Langerhans cells*

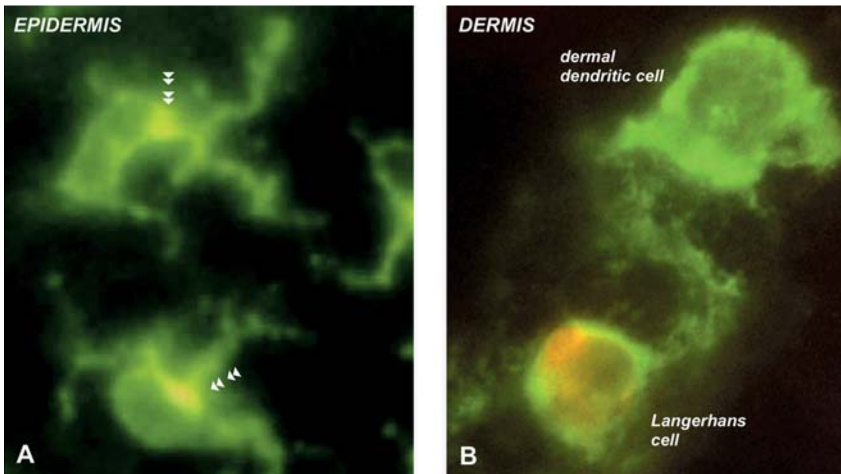

Fig. 5.4 Translocation of MHCII molecules to the cell surface during Langerhans cell maturation in the mouse. Immunofluorescence using anti-MHC II monoclonal antibody (green fluorescence) was performed on epidermal sheets of untreated skin (A) and on a dermal sheet of a whole skin explant that had been cultured for 48 h (B). The two cells in B are located in a lymph vessel ("cord"). The migrating Langerhans cell was additionally identified by its expression of langerin (red fluorescence). Even by conventional fluorescence microscopy it becomes evident that Langerhans cells *in situ* have most MHCII molecules concentrated in a bright intracellular spot (arrows in A). During migration and concomitant maturation most MHCII becomes translocated to the cell surface and thus appears as a brightly fluorescing rim. This applies both to the Langerhans cell and to the langerin-negative dermal dendritic cell travelling through the lymph vessel (B).

mature into potent immunostimulatory dendritic cells in vitro". The scenario whereby immature Langerhans cells take up and process antigen in peripheral tissues, mature and migrate to the lymph nodes and present the antigen there to T cells – although being true for Langerhans cells from skin [31, 93] or lungs [120] – was often thought to apply to all other dendritic cells in a lymph node. Wilson and Villadangos therefore called it the "Langerhans cell paradigm" [14]. As pointed out by these authors, recent developments suggest a less monolithic view on this topic.

5.8
The Changing of the Paradigm

The Melbourne group, inspired by its "father" Ken Shortman, did pioneering work in isolating, defining and functionally characterizing several distinct subsets of dendritic cells in the lymphoid organs [121, 122]. These observations suggested that the Langerhans cell paradigm may not be valid for all types of dendritic cells [14]. In mouse lymph nodes they found a majority of dendritic cells that were immature as measured by the lack or low expression of MHC II and co-stimulatory molecules as well as their ability to process protein antigens [99]. Thus these dendritic cells did not conform to the paradigm. It was proposed that they did not come into the lymph nodes via passage through a peripheral tissue such as epidermis, dermis, or lung. Rather, they may hypothetically develop from precursors in the blood or progenitor cells within the lymph node proper.

Furthermore, data by the Caux/Banchereau group pointed at important functional differences. Langerhans-like cells (langerin$^+$, E-cadherin$^+$, Factor XIIIa$^-$) were propagated from human CD34$^+$ hematopoietic stem cells and compared to interstitial-type dendritic cells (langerin$^-$, E-cadherin$^-$, Factor XIIIa$^+$) grown in parallel from the same source. The former were derived from a CD1a$^+$/CD14$^-$ the latter from a CD1a$^-$/CD14$^+$ intermediate precursor [28]. The most striking finding was that Langerhans-like cells failed to secrete IL-10 and to induce naive B cells to differentiate into IgM-secreting cells, in response to CD40 triggering and interleukin-2. Interstitial-type dendritic cells did so efficiently [123, 124].

Finally, even though the Birbeck granule had been an exclusive marker for Langerhans cells ever since its discovery, it was only until Sem Saeland's group identified langerin/CD207 as the granule's molecular correlate [1, 91] that we had good reasons to believe that this may also mean some Langerhans cell-specific functional features. Whereas the C-type lectin receptor langerin is expressed on Langerhans cells but not on other types of dendritic cells, the C-type lectin receptor DC-SIGN/CD209 shows the reciprocal expression pattern [125]. It may thus be possible that the spectrum of pathogens that is recognized by langerin$^+$/DC-SIGN$^-$ Langerhans cells and by langerin$^-$/DC-SIGN$^+$ dermal dendritic cells (or other types of dendritic cells) differs. Whether this is true remains to be seen. At present, much is known about pathogen-binding properties of DC-SIGN [126], but comparably little about those recognized by langerin [127, 128]. In addition, langerin is strikingly involved in recognition and routing of *Mycobacterium leprae* extracts,

leading to presentation to T cells in a CD1a-restricted fashion [129]. This may point to a specialized function of Langerhans cells in the responses to glycolipid antigens, by virtue of the sugar-binding properties and the unique subcellular routing of langerin (from the cell surface into Birbeck granules where it intersects with CD1a molecules).

These examples emphasize the distinct identity of Langerhans cells vis-à-vis other types of dendritic cells. It will be interesting to learn how these peculiarities of Langerhans cells relate to the *in vivo* situation.

5.9
Can Langerhans Cells Induce Immunity *in vivo*?

There is no doubt that Langerhans cells can sensitize naïve T cells very efficiently *in vitro*. Many studies over the years have buttressed this notion. They were all building up on the classical observations by Stingl, Katz, Braathen, Schuler and others who found that epidermal Langerhans cells possessed a strong immunogenic potential for helper as well as cytotoxic T cells [5, 130–133]. Also, the capacity to process proteins for the MHC class II pathway is well developed in immature Langerhans cells [105], and it also seems that they are efficient in crosspresenting exogenous antigens for the MHC I pathway [134]. In many situations Langerhans cells were found that carried pathogens *in vivo*, e.g. Dengue virus [135], tick-transmitted encephalitis virus [136], HIV-1 [137], or Leishmania parasites [138]. Moreover, much research has been performed in the contact hypersensitivity model where it is generally assumed that Langerhans cells are a critical antigen-presenting cell for initiating this reaction. This assumption is based on ample evidence that shows migration and antigen transport by Langerhans cells [29, 139, 140] or the dependence of the reaction on the density of Langerhans cells at the site of sensitization [141]. Highly enriched populations of Langerhans cells can also effectively sensitize when they are haptenized *in vitro* and injected into mice [142]. The late J. Wayne Streilein and Paul Bergstresser set the early milestones in this field.

It came therefore somewhat as a surprise to many Langerhans cell researchers when a number of recent reports cast some doubt on the notion that Langerhans cells would be equally efficient in inducing immunity *in vivo* [143]. Allan et al. [144] used a mouse model of epidermal infection with *Herpes simplex* virus. Interestingly, herpes-specific T cells in these mice were not activated by Langerhans cells, suggesting that they may not have picked up and transported the virus to the lymph nodes in this setting as one might have expected. Instead, a population of $CD8\alpha^+$ dendritic cells in the lymph nodes was found to present herpes peptides. On the other hand, when migration of skin dendritic cells was blocked, the T-cell response against herpes was not generated (R. Allan, personal communication). From these observations the authors develop a hypothesis which still assigns a critical role to Langerhans cells, perhaps in the efficient transport of antigen to the lymph nodes, though not in the direct presentation to T cells [145]. Another herpes virus model revealed that Langerhans cells of the vaginal epithelium do also not present viral

antigens to lymph node T cells. In that case, however, it was a CD8α-negative population of submucosal dendritic cells that carried and presented viral peptides [88].

As experimental models and methods for *in vivo* investigations become more refined the views on the role of Langerhans cells may change. This is also exemplified by studies in Leishmania infection models. There, it has previously been clearly shown that Langerhans cells are critical in that they transport Leishmania organisms from the skin to the lymph nodes [138]. Recent observations indicate that other types of dendritic cells, presumably dermal dendritic cells may be superior to Langerhans cells in that particular model [146]. Yet another example may be mentioned. Langerhans cells take up and harbor prion proteins [147]. In an *in vivo* infection model, however, Langerhans cells appear not to be involved in the transport of such an infectious agent (scrapie protein) to the lymph nodes [148]. It will be interesting to learn what future studies in these and similar models using a wide array of different antigens/pathogens will reveal. It will indeed be important to critically evaluate the different experimental models, and refrain from drawing premature conclusions as to the role of Langerhans cells “in real life”.

Finally, very recent experiments in mice where Langerhans cells can be selectively ablated, in other words “removed”, from the epidermis show that contact hypersensitivity develops almost at an unimpaired magnitude in the total absence of Langerhans cells [30, 31]. Even though a contribution of dermal dendritic cells to the generation of contact hypersensitivity has long been accepted [149] it was still surprising that Langerhans cells did apparently contribute so little to this reaction. Future experiments using different contact sensitizers over a range of different concentrations [150] will shed more light on this important issue.

In summary, the scientific controversy that was sparked off by the report of Allan and colleagues [144] and by the development of new mouse models [30, 31] will turn out to be highly fruitful in contributing to our understanding of the function of Langerhans cells *in vivo*. There is no doubt that this question will keep us busy for some time yet.

5.10 Can Langerhans Cells maintain Peripheral Tolerance in vivo?

Dendritic cells not only serve as potent inducers of immunity but also have an important function in establishing and maintaining peripheral tolerance [151]. This was shown in the past few years in a series of elegant experiments that used targeting of standard model antigens (ovalbumin, hen egg lysozyme) to the DEC-205/CD205 receptor on dendritic cells. When a co-stimulus (typically CD40 ligand) is co-administered with the antigen conjugate, the outcome is a vigorous T-cell response of both CD4 and CD8 T cells [152–154]. However, if antigen is targeted to DEC-205 in the absence of inflammation and co-stimulation, i.e. in the steady state, T-cell unresponsiveness ensues, involving both the CD4 and the CD8 compartment [152, 155]. From these observations it was concluded that dendritic cells

in the steady state constantly move from peripheral tissues and organs to the lymph nodes in order to tolerize against self antigens, that have escaped central tolerance, and against innocuous substances [151]. It is reasonable to assume that Langerhans cells in the steady state may also fulfill such a task, in particular, since it was convincingly shown that Langerhans cells in the steady state transport self antigens from the epidermis (melanin granules in that example) to the lymph nodes [156]. None of the above-mentioned studies, however, has addressed Langerhans cells. Two approaches have been taken lately in order to shed some light on this question.

Firstly, it was reasoned that Langerhans cells that migrate in the steady state may exhibit distinct phenotypical or functional properties by which they can be recognized. Using anti-langerin antibodies to identify Langerhans cells in the lymph nodes, we recently failed to detect gross phenotypical differences between those Langerhans cells that had entered the lymph nodes in the steady state and those that had arrived in response to skin inflammation [100]. Some slight but highly reproducible upregulation of expression levels of co-stimulator molecules (CD40, CD80, CD86, CD273, CD274) on Langerhans cells was noted, very similar to what has been observed with migratory lymph node dendritic cells in response to the administration of CD40 ligand [152, 155]. Ohl et al. [157] observed the same phenomenon on CD11c^{+}/MHC II^{high} populations in the lymph nodes that included substantial proportions of Langerhans cells. From these experiments it became also clear that the migration of Langerhans cells in the steady state requires expression of CCR7. Our own unpublished observations in clinically normal appearing human skin are in line with the data from mouse models. The very few Langerhans cells (defined by their langerin expression) that migrate through the dermis display phenotypical markers that are often associated with maturation (CD86, DC-LAMP, CCR7), but this does not necessarily prove full functional maturation (also see Chapter 1 by Steinman). In the mentioned studies it could not be determined whether Langerhans cells that migrate in the steady state induce tolerance. A major obstacle is our inability to physically sort and isolate Langerhans cells from the lymph nodes. New mouse models will enable this soon (see below and refs. [30, 31]).

The second approach to this question employs transgenic mouse models and chimeric mice. Important observations were made in transgenic mice, where the model antigen ovalbumin was expressed in the epidermis under a keratin promoter. It was asked whether Langerhans cells would tolerize against this antigen. When such mice were given transgenic, antigen-specific T cells ("OT-I" or "OT-II" cells) tolerance could not be established. Rather, Langerhans cells presented the antigen and a graft-versus-host like disease developed [158, 159]. Harnessing the fact that in bone marrow transplantation experiments Langerhans cells are long-lived [56] as well as the availability of mice with mutated MHC I molecules that cannot present the antigen ("bm8" mice), Mayerova et al. established chimeric mice where *only* Langerhans cells, but no other type of dendritic cell, possessed the correct K^{b} molecules that could present the ovalbumin peptide SIINFEKL to the transgenic OT-I cells. In the steady state these mice showed expansion of trans-

ferred transgenic T cells suggesting that no tolerance was induced [158]. Furthermore, when transgenic mice expressing the antigen only in the epidermis were crossed with transgenic mice that possessed the antigen-specific T cells in their lymphoid organs (OT-I mice) most mice died from the disease within a few weeks after birth. Tolerance did obviously not develop in this setting or it was too weak in order to stop the (unnaturally) many transgenic T cells from becoming activated and doing harm. This was in contrast to OT-I mice that were cross-bred with mice that transgenically expressed the antigen ubiquitously. These mice survived and did not develop disease because they had been rendered tolerant. In a similar model with transgenic expression of the ovalbumin antigen under a keratin promoter Shibaki et al. [160] also observed a graft-versus-host like disease when transgenic OT-I cells were adoptively transferred into these mice. If, however, the transgenic mice were allowed to develop normally (without any T-cell transfer) they were indeed tolerant, meaning that they could not be immunized as measured in a classical cutaneous delayed type hypersensitivity reaction (DTH). These data suggest that in the steady state during development of the animal, Langerhans cells may take up the antigen in the epidermis, migrate to draining lymph nodes and cross-present it there to antigen specific T cells in a tolerogenic fashion.

Clearly, more work is needed to gain a definitive picture about the tolerogenic potential of Langerhans cells *in vivo*. At present, there is no compelling evidence that would contradict such a role "in real life".

5.11
Can Langerhans Cells be Applied in Immunotherapy?

Clinical trials that attempt to treat cancer by administering autologous, *in vitro* grown dendritic cells "loaded" with tumor-specific antigens are being conducted and have so far yielded encouraging results [161–163]. Most studies are using dendritic cells grown from monocytes of the blood in the presence of the cytokines GM-CSF and IL-4 [164].

In contrast, there is only one important research group that has been employing CD34-stem cell-derived dendritic cells for clinical studies of immunotherapy for cancer. These cells contain low percentages of Langerhans cells [165], and the authors have published evidence that these cells may be particularly immunogenic [166–168]. More recent *in vitro* studies compared the immunogenic potential of Langerhans-like cells derived from $CD34^+$ stem cells with monocyte-derived dendritic cells in a side-by-side fashion, and found the former to be superior in the induction of antigen-specific CTLs [169, 170]. This emphasizes the need for more research addressing Langerhans cell-typical properties that may eventually be harnessed for immunotherapeutical purposes.

As an alternative to the adoptive transfer of dendritic cells or, for that matter, Langerhans cells, immunization via the surface of the skin (epicutaneous immunization) should be considered. Most standard vaccinations are in fact done via the skin: vaccines are injected into the skin, subcutaneously or intradermally. In con-

trast, in epicutaneous (sometimes also referred to as percutaneous or transcutaneous) immunization the antigen is applied topically onto the surface of the skin. Several studies in mouse models have shown that T-cell immunity, including the development of cytolytic T lymphocytes and protection from tumor cell challenge can be efficiently induced by epicutaneous immunization [171–174], even better as compared to the standard intradermal or subcutaneous routes [171, 175, 176]. For immunity to be generated it seems to be essential that an inflammatory milieu is present at the site of injection [171, 175], otherwise regulatory or suppressor T cells may be induced. The latter effect, however, may be desired in the treatment of autoimmune diseases where a critical (and not unexpected) involvement of Langerhans cells was also shown [173]. Clearly, this approach may be promising for clinical application. Before that, however, important questions need to be addressed and solved. How significant is the contribution of Langerhans cells as compared to other types of cutaneous dendritic cells (mainly dermal dendritic cells)? How can (tumor) antigen be delivered best to Langerhans cells *in situ*? Is it possible to exploit surface receptors on Langerhans cells, such as DEC-205/CD205 or langerin/CD207 for that purpose?

5.12
Recent Methodical Advances Relevant for the Study of Langerhans Cells

In vitro studies have indicated that several cell types have the potential to give rise to Langerhans cells. Dendritic cells grown from $CD34^+$ hematopoietic stem cells consistently contain Langerhans cells, as defined by Birbeck granules and the expression of langerin [165]. By selecting for specific subsets, e.g. $CD14^-/CD1a^+$ cells [28] or CLA^+ cells [177] one can enrich for Langerhans cells. Furthermore, the above sections of this chapter have emphasized the usefulness of antibodies against langerin to select and characterize the populations of Langerhans cells in epidermis/epithelia and in lymphoid tissue. Finally, in a recent review article from the year 2003 [6] we have foreseen the advent of tools such as mice that express the fluorescent marker EGFP under the control of the langerin promoter, similar to what had been established previously for CD11c [178]. This means that in these mice only Langerhans cells, but no other cell types fluoresce. This mouse model offers fascinating opportunities for “tracking & tracing” Langerhans cells along their pathways. It also enables researchers to highly enrich Langerhans cells from lymphoid organs by virtue of their langerin expression in the fluorescence-activated cell sorter (FACS). This was not possible up to now because cell surface expression of murine langerin, that is needed for any kind of antibody-mediated immunofluorescent or immunomagnetic sorting, is not equal to intracellular langerin expression [93]. Thus, one would inevitably have lost a substantial portion of the desired cells. Another transgenic model, in which expression of a cell death-mediating receptor (e.g. human or simian diphtheria toxin receptor in mice) is directed by the langerin promoter, allows one to selectively remove (ablate) Langerhans cells from the animals. Both above mouse models have very recently been established by two

research groups [30, 31]. A third and similar model where Langerhans cells are constitutively ablated has been described even more recently [179]. They will greatly contribute to our further understanding of Langerhans cell functions *in vivo*.

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6
Characterization of Dendritic Cells and other Antigen-presenting Cells in the Eye

Joan Stein-Streilein

6.1
Introduction

The eye is exposed directly to environmental pathogens on its surface and to blood borne pathogens in the internal compartments. Similarly to the brain, the eye resides behind blood–tissue barriers that are formed by endothelial cell tight junctions and other structural specialization, such as tight junctions among ocular pigment epithelial layers. While these barriers reduce the possibility of pathogen danger to the eye, they are not absolute and all types of infectious agents are known to cause eye disease. Thus, like other tissues and organs of the body, the eye needs to be defended by innate and adaptive immunity [1].

On the other hand, the visual axis of the eye focuses light images precisely on the retina which is intolerant of distortion that might be induced by innate or immune inflammation (Fig. 6.1). In addition, the corneal endothelium [2] and the neurosensory retina [3] are unable to regenerate if injured by trauma or inflammation. A phenomenon called ocular immune privilege, however, provides the eye with the necessary immune protection against infectious agents by allowing the expression of the least deleterious immune effector mechanisms [1]. Immune-privileged sites are defined operationally as sites in the body where foreign tissue grafts are capable of surviving for extended or indefinite periods of time. Of the ocular compartments that have been studied for immune privilege, tumor cell and antigen inoculation of the anterior chamber (AC) has been analyzed the most, but immune privilege extends to most if not all compartments of the eye [4–6]. Immune privilege was originally explained simplistically as the absence of lymphatic drainage and the creation of immunological ignorance for the organ [7]. However, the mechanisms of immune privilege are multiple and overlapping, and include both active and passive suppression of innate and immune inflammation.

The list of organs that share the privilege of the security of immune protection include the brain, the reproductive tract, the pregnant uterus, adrenal cortex, hair follicles and certain tumors. Similarly, tissues removed from the organs may also be privileged and include: in the eye: lens, pigment epithelium and retina; brain

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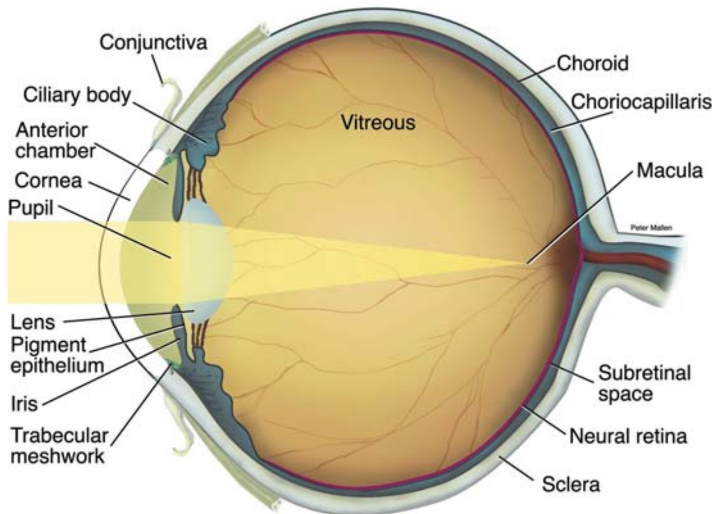

Fig. 6.1 Anatomy of the eye. The illustration identifies the anatomical parts of the eye and shows how light focuses on the macula or center of the retina.

and spinal cord; placenta and liver [8, 9]. The possibility is raised that some immune privilege exists in all organs.

Central to both immune response induction and the induction of tolerance (the outcome of immune privilege) is the antigen-presenting cell (APC). In general, the subset of APC that is uniquely well equipped for antigen presentation and is regarded as the sentinel of the immune response is the dendritic cell (DC) subpopulation [10]. T cells recognize antigens through interaction with APC that most of the time process, as well as present, antigen. APC include a heterogeneous family of cells that are able to process both exogenous and endogenous antigens into 10–20-amino-acid peptides, and load them on to MHC molecules, that then traffic to the membrane where they can be recognized by the antigen-specific T-cell receptor [11]. APC are further classified into professional APC (bone-marrow-derived DC) that are capable of activating and inducing clonal expansion of both naïve and memory T cells and nonprofessional APC (B lymphocytes, monocytes, macrophages, endothelial cells) that are able to stimulate memory T cells but are poorly equipped to stimulate naïve cells. Within the tissues, the DC phenotype is immature but is capable of maturation if presented with “danger” signals [12, 13]. Besides expression of co-receptors (CD80, CD86, Ox40 ligand, CD40) mature DC exhibit decreased endocytosis of extracellular antigens, translocate the peptide-loaded MHC molecules into the plasma membrane and display long-lasting peptide MHC complex. Mature DC also display increased membrane expression of chemokine receptor CCR7 [11] that responds to the stromal chemokines from the T-cell areas of the secondary lymphoid organs. In immune-privileged sites, the process of immune activation is regulated in part by interfering with the maturation of the DC and by altering the indigenous APC toward the induction of tolerance.

The requirement for protection against immune inflammation in the eye leads to regional specialization of the APC [14]. The initiation of the immune response or immune regulation begins with the indigenous APC that picks up the antigen and carries it to the draining secondary lymphoid organ where it may present the antigen or pass off its antigen to the APC in the region of the lymphoid organ where it finds itself. The aims of this review are to characterize the APC in different parts of the eye, and to explore the mechanisms used by the eye-derived APC in the development of peripheral tolerance induced following antigen inoculation into the anterior chamber of the eye. Knowledge of mechanisms used by APC in immune-privileged sites may be shared by APC in tissues elsewhere within the organism and therefore may be relevant to the induction and maintenance of self-tolerance in the adult and the prevention of autoimmunity.

6.2 APC in Various Regions of the Eye

It is known that the intra-ocular fluids of the eye [aqueous humor (AqH) and vitreous humor] contain biologically relevant concentrations of various immunosuppressive neuropeptides, cytokine, growth factors and soluble cell-surface receptors that interfere with the development of immune reactivity [15]. Aqueous humor inhibits innate immune effector cells [16, 17] but most important for our discussion, AqH modulates the antigen presenting capacity of the APC in eye [18–21]. Experiments have shown that ocular fluids remains immunosuppressive and anti-inflammatory even in eyes that are inflamed and under autoimmune attack, but the spectrum of responsible factors can change [22–24]. The fluids from the non-inflamed eye contain an abundance of latent TGFβ2 while the fluids from the inflamed eye contain activated TGFβ2. This is in part because "danger" signals (TNFα, IL-1) from the inflammation up regulate IL-6 production by the parenchymal cells, that in turn activate macrophages and the molecules that convert latent TGFβ to active [24]. It follows that the immunosuppressive fluids in other regions of the eye would have similar effects on the APC as they do in the front of the eye.

6.3 DC/APC in the Retina

The retina, also an immune-privileged site, can be a target of induced autoimmune disease. Potential APC are its $CD45^+$ (a marker of bone-marrow-derived cells) cells [25] (Fig. 6.2). The barrier formed by the retinal vascular endothelium and the retinal pigmented epithelium (RPE) provides a degree of sequestration, limiting the circulation of resting T cells (even those specific for a retinal antigen) through the uninflamed retina. [26]. Whether cells with DC-like properties sample the immunologically-quiescent retinal microenvironment is unclear. At present the most likely candidate for a retinal DC equivalent may be the perivascular cell (PVC) [27].

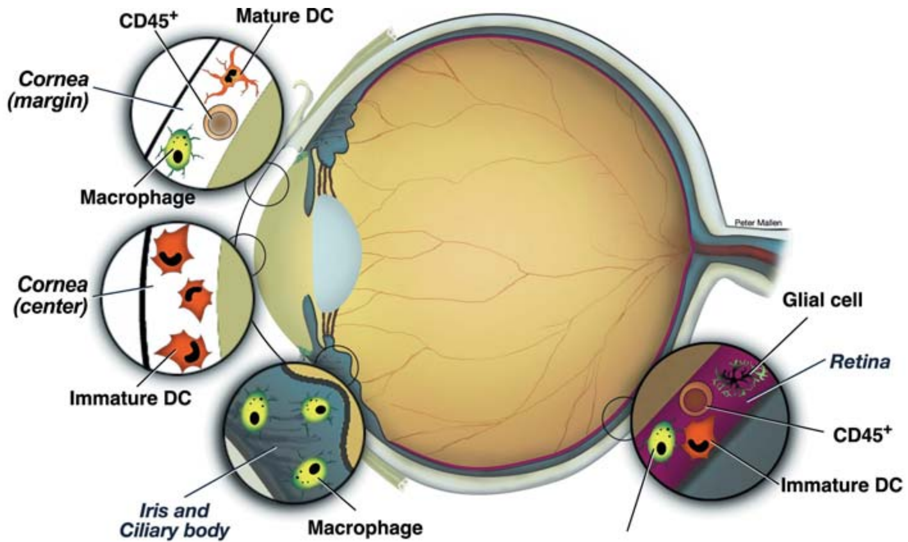

Fig. 6.2 APC in the eye. The illustration identifies the cells in the eye by marker and location. Retina: CD45+ and CD45+ CD11b, F4/80+ cells have been studied in the retina. Dendritic like cells have not been observed in this region. Cornea: A variety of potential APC are in the cornea and differentiated by their expression of surface markers and their location in the various regions of the cornea.

The immunoregulatory or peripheral tolerance process could be the outcome, following the recognition of retinal antigen delivered to lymphoid tissues outside the retina, as well as local recognition of retinal antigen on APC programmed to inhibit T-cell responses [28]. The CD45+ cells studied in the retina by Gregerson and Yang were CD45 intermediate, CD11b+ F480+ microglia [25]. They and others found that retinal microglia were relatively insensitive to the developing inflammatory environment probably because of the environment rich in TGFβ [29]. However, while retinal microglia represented the most numerous APC population in the retina, a small population of cells with PVC-like properties were also identified. The PVC-like cells expressed CD45 hi CD11c+, elevated MHC class I and were responsive to antibody stimulation through their CD40 co-receptors. No conventional DC were found in the retina. The effects of IFNγ and anti CD40 treatment were attenuated in the retina *in vivo* and in cultured CD45+ cells. In another report, Gregerson and colleagues demonstrated that indeed the CD45+ cells isolated from immunologically-quiescent murine retina have limited antigen-presenting ability [30]. Because the retina lacks tissue equivalents of meninges and choroids plexus, rich sources of DC in the brain, cells from retina may better represent the APC activity of fresh, adult CNS parenchymal and PVC. Thus, the activity of the retinal CD45+ cells appears to be directed toward limiting T-cell responses. It is reassuring to note that similar cells have been found in the human retina [31].

6.4 DC/APC in the Cornea

MHC Class II+ DC are resident in the area between the vascularized conjunctiva and avascular cornea called the limbus. Cells are recruited into the cornea from this population during inflammatory or pathological conditions [32–39]. It was believed until recently that the cornea was devoid of APC and other bone-marrow-derived cells and that lack of such cells contributed to the immune-privileged nature of the cornea. However, the cornea actually contains a resident bone-marrow derived CD45+ population. Hamrah showed that the CD45+ cells were CD11c+ CD11b– Langerhans cells [40–42]. In addition a population of CD45+CD11b+ CD11c– monocyte cells has been identified in the corneal stroma [42, 43], At the anterior portion of the corneal stroma a population of myeloid CD8a– monocytic (CD11b, CD11C+) DCs exist, that are ultrastructurely distinct from the monocytic CD11c– population in the posterior portion of the cornea. A less differentiated CD14+ cell appears throughout the corneal stroma. The fact that the APC in the central cornea lack both Class II and co-stimulatory molecules explains the original misconception that the cornea was devoid of DC or APC [36]. In contrast to the bone-marrow-derived cells in the central cornea about half of the CD45+ cells in the periphery and limbus express Class II. Furthermore, cornea allograft experimentation revealed that donor APC upregulate their Class II in the graft–host interface, the area of marked inflammation [40, 44] and later appear in the draining lymph nodes [45]. While the cornea is lymphatic-free, lymphatic vessels grow during inflammatory conditions [46–48, 113]. It is thought the cornea DC express VEGFR 3 and might respond to the lymphatic growth factor VEGF 3 and VEGFR3+ DC have been seen congregating around budding lymphatics [49, 50]. In another report, Maruyama et al. show that the DC surrounding the nascent lymphatics are CD11b+, CD11c– and that this innate cell may form new lymphatics in an unusual manner, distinct from angiogenesis [113].

In summary, the noninflamed cornea possesses a variety of APC including immature or Class II negative DC that are capable of maturation given the right signals. The markers on the APC in the cornea vary in their expression of surface markers [49, 51] and in many instances cannot be classified definitively into macrophage or DC subcategories. With the exception of the CD11b+CD11c– cell, the function of each of the many APC/DC subpopulations described to be in the cornea remains to be determined.

6.5 DC/APC in the Anterior Chamber

The APC in the anterior uveal tract have been described by several studies [52, 53]. Toll-like receptors (TLR) are part of the innate immune system that, when bound by molecules expressed by invading bacteria, signal activation and immune inflammation [54]. The expression of TLR has not been well studied in the eye of the

mouse. However, Chang et al. reported that resident APC of the human uvea express HLA-DR and TLR-4, and its associated LPS receptor complex CD14 [55]. Since, in their opinion, the TLR-4 MD-2+ APC were HLA-DR+ DC, they therefore suggested that gram-negative bacteria might be involved in the pathogenesis of acute anterior uveitis.

In the anterior chamber associated immune deviation (ACAID) model for immune privilege, the F4/80+ cells from the iris and ciliary body pick up antigens administered to the front of the eye by anterior chamber (a.c.) inoculation [56]. Recently, Camelo et al. reported that the type of APC that carried antigen from the eye of rat after intracameral injection of antigen, was, as in the mouse, predominantly resident macrophages, negative for Class II but that appeared on histological examination to be not only in the iris but in all tissues lining the AC of the eye [57]. The resident APC lie mainly within the iris and ciliary body and perhaps in the cornea. Post a.c. inoculation the resident F4/80+ population takes up the antigen, moves out through the trabecular meshwork into the blood and travels to the spleen. In fact removal of the spleen prevents the induction of tolerance (ACAID) through the eye [58]. Dullforce and colleagues confirmed the generally accepted notion that a.c.-inoculated antigen was taken up by APC that traveled to the spleen by showing that eye-derived APC *did not travel* to the lymph nodes [59]. Since lymphatics or nascent lymphatics are normally present only in the conjunctiva [60, 61] or in the inflamed cornea [62, 63], it is not surprising that antigen-transporting APC do not travel to the draining lymph nodes.

It is not unreasonable to assume that since 98% of the antigen inoculated into the AC goes directly into the blood, ACAID is merely i.v.-induced tolerance. However if the investigator bypasses the eye and inoculates the same amount of antigen directly into the blood, ACAID-type tolerance is not induced [64–66]. Supposedly, transplanted allogeneic corneas that abut the anterior chamber would have their antigens picked up by eye-derived APC and/or delivered to the spleen by donor APC in a similar manner. Sonoda et al. showed that mechanisms that induce ACAID allow for prolonged cornea graft survival in a mouse model [67].

As stated above, AqH is an immunosuppressive fluid in the anterior chamber and therefore one might expect that the APC bathed in immunosuppressive molecules would have a distinct phenotype [18, 21]. The APC indigenous to the anterior portion of the eye are neither immature nor mature dendritic cells. To fit the APC of the AC into the procrustean bed of DC classification the "eye-derived" APC could be categorized as semi-mature DC [10]. Characteristically the "eye-derived" APC share markers with dendritic cells and macrophages. It is not clear if the APC indigenous to the anterior uveal tract are DC with a special phenotype, or macrophages with a special phenotype.

The F4/80 cell was shown by Wilbanks to be the cell-associated signal from the eye [68, 69] that traveled through the blood to the spleen to induce ACAID. The F4/80+ cell lacks Class II, does not express traditional co-receptors for immune activation (CD40 and IL-12) and produces IL-10 and activated TGFβ [56, 70–72]. Both IL-10 and TGFβ are monocrines, capable of inducing their own secretion and thereby contributing to the forceful influence that eye-derived F4/80 cells have on the functional phenotype of APC/DC they meet in the periphery.

The ACAID F4/80 cell also produces unique inflammatory chemokine profiles. The F4/80+ APC produces MIP-2 but not other inflammatory chemokines [66, 73, 74]. The MIP-2 chemokine is capable of recruiting CXCR2+ natural killer T (NKT) cells to the spleen. Furthermore, The ACAID F4/80 cell expresses CD1d and it is known that CD1d interaction with the invariant (i)TCR on the NKT cell is crucial for the induction of ACAID and peripheral tolerance.

Since it is next to impossible to obtain sufficient resident F4/80 eye-derived APC, the expression of genes in ACAID-like APC has been analyzed with surrogate ACAID F4/80 APC [20, 75] by two laboratories. Masli studied a macrophage hybridoma #59 [76, 77] treated with TGFβ and antigen and Zhang-Hoover explored the genes in bone-marrow-derived F4/80 APC generated with L929 supernatants (contains MCSF) [114]. Both investigators found that the genes that support IFNγ- and NF*k*B-dependent immune reactivity were downregulated, while the genes that promote or are involved in TGFβ function were upregulated. Extending the genetic studies, Masli published on the role of thrombospondin in TGFβ activation and ACAID [77], while Zhang-Hoover focused experimental studies on chemokines and their receptors and traffic of the F4/80+ APC to the splenic marginal zone (MZ) [114]. The F4/80 ACAID APC most probably do not move into the T-cell areas because the critical chemokine receptor that is required for moving into the T-cell area, CCR7, is expressed at only very low levels or not at all [114]. However, The F4/80 ACAID APC also lacks the chemokine receptor that identified immature APC in the tissues (CCR6). Thus, studies of modulated genes in ACAID-like APC contributed novel and different information about APC in immune-privileged sites, such as the eye.

6.6 Mechanisms of ACAID Induction in the Spleen

Functional studies involving the APC in the uveal tract have been a product of the investigations on mechanisms of immune privilege and ACAID. As stated above, ACAID is a deviant state of immunity that is responsible for the induction of peripheral tolerance to both self and foreign antigens that arrive via the eye [1, 78]. While Wilbanks and colleagues showed that the F4/80+ cell carried the tolerogenic signal to the spleen, and Niederkorn reported the importance of B cells in ACAID induction [79, 80], Sonoda et al. were the first to show that the "eye-derived" APC not only interacted (in the spleen) with the T cells that were to become T regulatory (Treg) cells but necessarily interacted with a rare lymphocyte called the iNKT cell. The NKT cell bears markers of both natural killer cells and the traditional T cell. Of the NKT cells, 85% express the invariant Vα14Jα18 TCR that preferentially binds a few Vβ chains. The murine iNKT cell has a counterpart in the human that expresses the Vα24 JQα [81–83]. The TCR on the NKT cell is oligoclonal and interacts (presumably) with foreign or self lipids presented by the class I-like molecule called CD1d [83–86]. In the mouse the iNKT cell may be either CD4+ or DN and it is suggested that the CD4+ iNKT cell produce IL-4 and IFNγ while the

DN are mainly producers of Th1 type cytokines. We know that during ACAID induction, the required iNKT cell is CD4+ [87], secretes IL-10 and not IL-4 [73].

The eye-derived APC that are transporting antigen to the spleen also recruit iNKT cells to their splenic destination by the release of MIP-2 [66]. This has been confirmed by microgene analysis [77]. Unexpectedly, the destination of the blood-borne cells turns out not to be the traditional T-cell areas, where most adaptive immune cells are destined to meet, but the marginal zone (MZ) (Fig. 6.3). During an adaptive immune response CCR7+ APC and other CCR7+ precursor cells that arrive in the spleen via the blood are "poured" from the central arteriole into the MZ but leave within 4–6 h to follow their chemokine gradient to the T-cell areas. Other cells that are CCR7 negative move into the red pulp and are degraded. Faunce et al. convincingly showed that the aggregates that contain the F4/80+ APC, T cells, and NKT cells are in place in the MZ as late as 7 days post the a.c. injection [66]. B cells are also required for the induction of ACAID [80, 88]. Sonoda showed that the subset of B cells required for ACAID is the CD1d+ MZ B cell [89]. Niederkorn's group suggests that the antigen transporting APC from the eye may "hand over" its antigen to B cells in the spleen for a required antigen presentation by the B cell via Qa-1 (a MHC Class 1-B molecule) [90] to the CD8 T cell [91, 92].

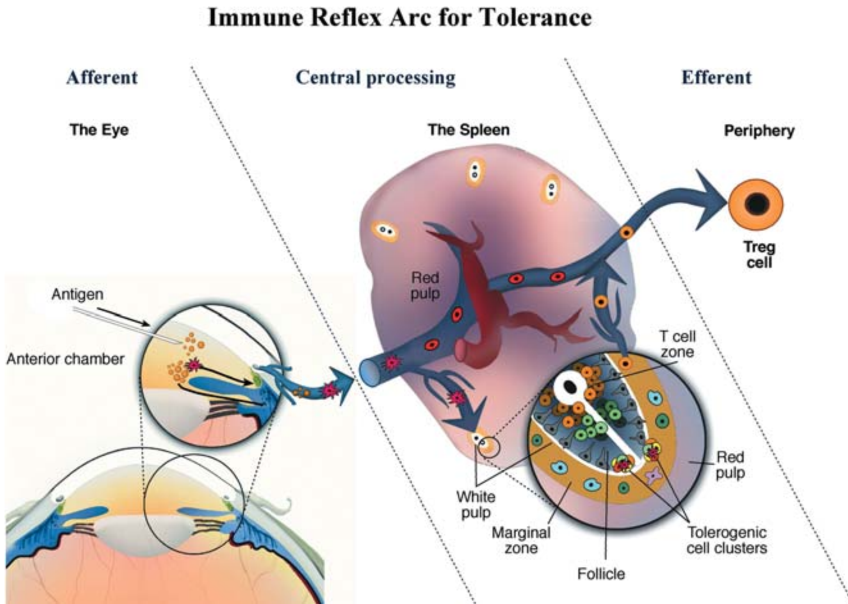

Fig. 6.3 Immune reflex arc of tolerance. The illustration shows three stages of mechanisms involved in the induction of peripheral tolerance in ACAID. Antigen is presented to the anterior chamber, where it is picked up by the F4/80+ APC located in the trabecular meshwork, ciliary body or the cornea. The antigen-loaded APC then leave the tissue via the trabecular meshwork and travel to the blood to the marginal zone of the spleen where they interact with other cells to produce Treg cells. Treg cells then travel to the periphery where they function as afferent and efferent T suppressor cells.

6.7 The Role of the F4/80 Protein in ACAID

F4/80 is the molecule that is recognized by an antibody that was developed by Siamon Gordon's group more than 20 years ago, [93]. F4/80 is a prototypic member of the EGF-TM7 receptor family that includes EMR1, 2, 3, 4, ETL and CD97 [94, 95]. A dual adhesion and signaling function has thus been suggested for the EGF-TM7 molecules where the extracellular region is involved in protein–protein interaction with other cell surface proteins and/or extracellular matrix proteins, triggering intracellular signaling through the TM7 domain. Consistent with this hypothesis, specific cellular ligands for the EGF-TM7 receptors have been reported. CD55 (decay accelerating factor, DAF) was identified as the cognate cellular receptor for CD97 [96]. The F4/80 molecule has been established as one of the most specific markers for murine macrophages. F4/80 is highly and constitutively expressed on most resident tissue Mϕ populations such as the red pulp Mϕ in the spleen, microglia in the brain, Kupffer cells in the liver and Langerhans cells in the skin [95]. Furthermore, the expression of F4/80 is tightly regulated according to the physiological status of cells. Thus, the precursor of tissue Mϕ, the blood monocyte, is known to express less F4/80 than its mature counterparts [97]. F4/80 is expressed at lower levels on activated Mϕ isolated from Bacille Calmette–Guérin (BCG) infected animals in comparison to unstimulated resting Mϕ [98]. Similarly, F4/80 expression is downregulated on Mϕ in response to interferon-γ [99]. F4/80 expression on Langerhans cells decreases after they take up antigens and become migrating dendritic cells; in lymph nodes and spleens. Since F4/80 is detected only on Mϕ in T-cell independent areas [97] the fact that the ACAID cells aggregate in the MZ rather than the T-cell areas of the spleen is not surprising. These studies point to a specialized function for F4/80 protein on tissue Mϕ populations.

The early studies involving the ACAID model showed that F4/80 was a marker of the eye-derived cell that carried the tolerance-inducing signal to the spleen. It has been shown that ACAID can be induced in naïve mice with the adoptive transfer of as few as 20 F4/80+ APC generated *in vitro* by treatment with TGF-β2 and antigen [20]. Early studies showed that F4/80 antibody given *in vivo* prevented the suppression of DH (ACAID) in experimental mice [69, 100]. However, the mechanism of the antibody treatment or the role of F4/80 protein in the model was not studied further until recently [101]. Suppression of DH response following a.c. inoculation of antigen is caused in part by the splenic CD8+ efferent Treg cells that develop. The efferent Treg is capable of suppressing both effector T-cell antigen-specific Th1 [56] and Th2 responses [102]. With the advent of the F4/80 knock-out mouse the function of the F4/80 protein was testable. When the splenic T cells were harvested from the a.c.-treated mice, the T cells from the WT mice were able to suppress adoptively transferred DH responses but the T cells from the F4/80 knock-out mice were not. Therefore, it was concluded that peripheral tolerance (ACAID) that developed subsequent to a.c. inoculation of antigen failed in F4/80$^{-/-}$ mice due to a lack of CD8+ efferent Treg-cell development [101]. Thus the F4/80 protein plays a role in the development ACAID in part by facilitating the develop-

ment of the CD8+ Treg cell. Similar to the ACAID model, a low dose oral tolerance model in mice also generates CD8+ Treg cells capable of suppressing Th1 effector functions [103]. In addition, like the indigenous cells in the eye, some indigenous Mϕ in the Peyer's patches express the F4/80 Ag [104]. Due to the similarities in generation of efferent CD8+ Treg cells in two tolerance models, Lin and colleagues postulated a direct role for the F4/80 molecule in the induction of peripheral tolerance and showed that F4/80 expression was also required for the induction of CD8+ Treg in an OVA model of oral tolerance [101].

6.8 Therapeutic Potential of ACAID APC

The possibility that ACAID might be used therapeutically was obvious in much of the original work with eye-derived APC. In the late 1980s, Streilein's group showed that induction of ACAID through a.c. inoculation altered the induction and progression of autoimmune uvietis [105] and later this group showed that a.c. inoculation of antigen altered Th2-dependent pulmonary pathology [78, 102]. Directly related to these studies is the report by Kezuka et al. that ACAID mechanisms are functional in humans [106]. Surrogate ACAID APC have been used by several groups to evaluate the mechanisms of ACAID [20, 107, 108] and to explore the genes that make these F4/80+ cells tolerogenic [56, 77, 78, 109]. It was only a small leap of faith that ACAID-like APC could be used to modulate immuno-inflammatory conditions in other familiar animal models. Therapeutically it is important that ACAID can be imposed on the presensitized state [110]. Faunce and colleagues reported that APC exposed to TGFβ2 and myelin basic protein (MBP) were able to induce peripheral tolerance to that antigen when inoculated into naïve or previously sensitized C57 BL/6 mice. The MBP-specific tolerogenic APC decreased both the severity and incidence of ongoing EAE [111]. Furthermore the tolerance could be adoptively transferred from the suppressed mice to naïve mice with CD8+ cells but not CD4+ T cells. Similar results are reported for treatment of an autoimmune model of Th1 mediated pulmonary interstitial fibrosis induced by a hapten [112]. Thus lessons learned from understanding mechanisms of immune privilege and the mechanisms of regional specialization used by its indigenous APC may be applicable to other regions of the body both for understanding tolerance induction to self and foreign antigen and for development of therapeutic maneuvers that will regulate unwarranted immune responses and restore of tolerance to specific antigens through out the body.

6.9
Conclusions and Implications

The eye possess specialized functions that contrive to protect vision and maintain a noninflammatory state of immune privilege. "Eye-derived" APC protect the visual axis of mammals that live and see in a very narrow band of light waves. The phenotype of the eye-derived APC is modulated in part by the immunosuppressive fluids within the eye in such a way that they not only express the necessary co-receptors necessary for activation of immune responses but they also possess novel capabilities that actually prevent immune responses. Rather than tolerance being induced by the absence of signals to produce immune responses, tolerance is an active phenomenon that requires specialized signals and trafficking of cells that regulate both induction and expression of Th1 and Th2 immune responses [102]. The eye-derived APC prevent immune responses in part by recruiting specialized cells to novel regions of the spleen away from the T-cell areas to induce antigen-specific peripheral tolerance and T regulatory cells. It appears that a master substance in the aqueous humor of the eye is TGFβ2 and that TGFβ2 (or TGFβ1) can be used *in vitro* to induce surrogate ACAID-like APC. Current knowledge suggests that ACAID-like APC possess all aspects of a.c.-induced ACAID APC. Such *in vitro*-generated APC clearly induce antigen specific peripheral tolerance following their i.v. inoculation into naïve or sensitized mice. The subpopulation of tolerogenic APC in the eye is required to express the classical macrophage F4/80 marker if they are to effectively induce efferent CD8+ Treg cells *in vivo* or *in vitro*. The possibility is raised that F4/80 expression is a marker of tolerogenic APC in general. It is interesting that two routes of antigen administration (eye and gut) that induce peripheral tolerance and an efferent CD8+ Treg cells, both require the regional APC to express F4/80 protein. The question of how common the mechanisms involved in F4/80+ APC-induced tolerance used by the eye and immune-privileged sites are to other regions of the body is not known. One might predict that F4/80 APC indigenous to tissues and organs where regional specialization is required to subdue immune responses would possess tolerogenic traits similar to the eye-derived F480/APC.

Acknowledgements

This chapter is dedicated to the memory of my husband and colleague, J. Wayne Streilein who began his studies of immune privilege under the mentorship of Rupert Billingham in the 1960s, developed and studied the model of immune privilege in the eye called ACAID and continued to explore immune privilege in the eye throughout his life. I think of Wayne as the modern founder of Ocular Immunology and without his work I could not have written this chapter.

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Abbreviations

a.c.	anterior chamber (adj.)
ACAID	anterior chamber associated immune deviation
APC	antigen presenting cell(s)
AqH	aqueous humor
DC	dendritic cell(s)
MBP	myelin basic protein
MZ	marginal zone
NKT	Natural Killer T
PVC	perivascular cell
RPE	retinal pigment epithelia
RPE	retinal pigmented epithelium
TLR	Toll-like receptor
Treg	T regulatory cell(s)

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7
Toll-like Receptors

Hubertus Hochrein and Hermann Wagner

Dendritic cells (DC) are the central sentinels of the immune system which initiate innate as well as adaptive immune responses. To detect pathogens or other forms of danger DC are equipped with a variety of pattern recognition receptors (PRR). Amongst the PRR the Toll-like receptors (TLR) have gained outstanding attention in recent years.

7.1
TLR and their Ligands

Since the initial discovery of the *Drosophila* protein "Toll" more than 10 different, homologous proteins with highly-conserved extra- and intracellular domains have been identified in mammals, and have been named Toll-like receptors (TLR). Structurally, TLR are typical type one receptors with an extracellular domain consisting of leucine-rich repeats (LRR), one short membrane-spanning region and an intracellular domain. The latter is highly homologous with the respective counterparts of IL-1 receptor and Toll and thus was named the Toll-IL-1 R (TIR) domain. In contrast to the *Drosophila* Toll, where an endogenous ligand (Spaetzle) binds to the LRR, the LRR domains of TLR directly bind invariant "pathogen associated molecular patterns" (PAMPS) expressed by virtually all classes of microorganisms, including bacteria, viruses, fungi and parasites.

Table 7.1 shows a selection of TLR ligands sorted by their origin. TLR2 in combination (heterodimerization) with TLR1 or TLR6 recognizes a variety of lipoproteins, lipopeptides or polysaccharides of bacterial, viral or fungal origin [1]. TLR3 seems to be highly specific for double-stranded (ds) RNA, which might be either genomic viral RNA or dsRNA intermediates produced during viral replication [2]. TLR4, the first mammalian TLR identified, is the main receptor for bacterial derived lipopolysaccharides [3] and in addition senses some viral envelope proteins [4]. TLR4 may also act as a receptor for the plant-derived molecule Taxol® [5] and stress-induced molecules like the heat shock proteins [6]. TLR5 recognizes flagellin [7]. TLR7 and TLR8 represent receptors for single stranded RNA (ssRNA) from

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Tab. 7.1 TLRs and their ligands.

TLR	***Origin of ligands***					
	bacterial	***viral***	***fungal/ plant***	***parasitic***	***endogeneous***	***synthetic***
TLR1+2 TLR2+6	lipoproteins, lipopetides, peptidoglycan, lipoteichoic acid some LPS	hemagglutinin gB of HCMV	zymosan β-glycan	glycosyl-phosphatidyl-inositol (*Trypanosoma*)	HSPs	synthetic lipopeptides (Pam3Cys)
TLR3		g-dsRNA, dsRNA inter-mediates				poly(I:C)
TLR4	LPS, HSP	protein F of RSV Env of MMTV	Taxol		HSP60, HSP70, fibrinogen, fibronectin, oligosaccharides of hyaluronic acid, dectin, uric acid	
TLR5	flagellin					
TLR7 TLR8		g-ssRNA			vertebrate RNA (in complex)	poly U-RNA, poly GU RNA, guanosine analogs (imidazoquinolines TLR7 and TLR8, loxoribin TLR7 only), siRNA
TLR9	gDNA, pDNA	gDNA		hemozoin (parasitic modified hemoglobin), parasite DNA? (malaria, leishmania)	chromatin-IgG complexes, vertebrate DNA (in complex with cationic lipids),	CpG-ODN (A, B, C type), non-CpG-ODN (in complex) synthetic hemozoin
TLR10	not defined	not defined	not defined	not defined	not defined	not defined
TLR11	uropathogenic bacteria					

Abbreviation: g: genomic; ss: single stranded; ds: double stranded; p: plasmid;
gB: glycoprotein B; Env: envelop protein; HSP: heat shock protein;
ODN: oligodeoxynucleotide; HCMV: human cytomegalovirus; LPS: lipopolysaccharide;
MMTV: mouse mammary tumor virus.

RNA viruses like influenza or HIV [8, 9]. Synthetic ligands for TLR7 have also been identified. Examples include guanosine analogs like loxoribin and resiquimod, the latter additionally acting through human TLR8 [10–12]. Originally TLR9 was described as a receptor for CpG motif containing unmethylated bacterial or synthetic DNA [13, 14]. Recently the spectrum was broadened to include viral DNA from viruses of the herpes family, including HSV-1, HSV-2 and MCMV [15–18] or *Baculovirus* [19]. Hemozoin, a digestion product of haemoglobin by the malaria parasite has been recently added to the list of TLR9 ligands. Hemozoin is until now the only non-nucleic acid ligand for TLR9 [20].

For many years it had been assumed that TLR ligands bonded directly to their respective receptors, based on interpretation of functional data implying structural and species specificity of ligands for TLR4 and TLR9, an example being the species and sequence specificity of phosphorothiolated CpG containing oligonucleotides (CpG-ODN) to murine or human TLR9 [14]. Recent biochemical binding studies with TLR2, TLR4 and TLR9, however, have proven that PAMPS bind directly to TLR and thus do indeed act as ligands for their respective TLRs [21].

The definition of TLR-ligands as PAMPS is somewhat misleading because TLR-ligands originate from pathogenic and nonpathogenic organisms alike. An obvious example is the plant-derived TLR4 ligand Taxol. In addition, even though recent reports for host-derived endogenous TLR ligands (especially for TLR2 and TLR4) need to withstand time due to the potential of contamination with microbial products, the existence of endogenous TLR ligands as shown in Table 7.1 can no longer be questioned. The identification of vertebrate DNA as a TLR ligand is a prime example. Under nonpathological conditions vertebrate DNA has almost no stimulatory potential, a finding explained by suppression and methylation of CpG motifs within mammalian DNA. However, under pathological conditions as for example in systemic lupus erythematosus (SLE), immune complexes consisting of vertebrate DNA and anti-DNA antibodies strongly trigger TLR9 signaling, followed by B cell or plasmacytoid DC (pDC) activation [22, 23]. Experimentally this can be mimicked by complexing vertebrate DNA with cationic lipids. In this setting nonstimulatory DNA sequences (containing DNA without or with completely methylated CpG motifs) reveal upon endosomal translocation high TLR9-dependent stimulatory potential [24]. Thus vertebrate DNA can be added to the list of endogenous TLR ligands. Apart from DNA, immune complexes containing RNA and antibodies are also stimulatory (most likely through a TLR7/TLR8 dependent pathway) and are therefore included in Table 7.1.

7.2 TLR Subfamilies

TLRs can be categorized by means of amino acid sequence homology, cellular location, use of adaptor molecules or the nature of their specific ligands. TLR7, -8, -9 are now grouped together into the TLR9 subfamily because they share a high amino acid sequence similarity, cellular location in endosomes, the need for endo-

somal acidification for ligand binding and a structural relationship amongst their ligands (nucleic acids) [25]. The endosomal location (at least in DC) and the recognition of nucleic acids is also an attribute of TLR3. However, its amino acid homology to the members of the TLR9 subfamily is relatively poor. Thus TLR3 is seen as the sole member of its own group. Members of the TLR2 family (TLR1, -2, -6) or TLR4 or TLR5 are located at the extracellular membrane and recognize extracellular TLR ligands. However it has been reported that upon activation TLR2 becomes recruited to phagosomes. Thus even cell-membrane bound TLRs might change subcellular distribution upon stimulation.

7.3 TLR and Dendritic Cell Subsets

As described in detail by others (see Chapter 12) DC can be distinguished by phenotypic, functional or developmental means. Here we will focus on the different TLR expression pattern within DC subsets and the respective functional consequences of TLR ligation. A broad and simplified classification of DC subsets represents the separation into plasmacytoid (p) and conventional (c) DC [26]. In human and mouse, pDC preferentially express TLR7 and TLR9 and thus respond to the corresponding ligands (imidazoquinolines s.s.RNA and s.s.CpG-DNA) but not to ligands for TLR2, TLR3, TLR4 or TLR5 (reviewed in [27]).

In contrast, *ex vivo* isolated human c-DC as well as *in vitro* generated monocyte derived DC (MO-DC) express all TLR with the exception of TLR9. This particular TLR expression pattern by human c-DC translates into responsiveness to TLR2, -3, -4, -5, -7 and -8 ligands but a lack of responsiveness to the TLR9 ligand CpG-ODN. [28].

In the murine system TLR expression amongst DC is more complex, probably due to better separation techniques of conventional DC. Three major subsets of the murine spleen DC can be distinguished: $CD8^+$ ($CD8^+/CD205^+/CD4^-/CD11b^-$), $CD4^+$ ($CD8^-/CD205^-/CD4^+/CD11b^+$) and $CD4^-/8^-$ ($CD8^-/CD205^-/CD4^-/CD11b^+$). Whereas the $CD4^-/8^-$ subset expresses the complete panel of TLR 1–9 the $CD4^+$ DC subset shows only minimal expression of TLR3. In contrast, the $CD8^+$ DC subset shows an extremely high expression of TLR3, minimal expression of TLR5 and completely lacks the expression of TLR7. Since murine TLR8 is apparently not functional [11] the lack of TLR7 expression corresponds directly with unresponsiveness of the $CD8^+$ DC subset to the synthetic TLR7 and TLR8 ligand resiquimod [29]. Similarly to *ex vivo* isolated DC subsets, GM-CSF or Flt3-ligand-driven murine bone marrow culture-derived $CD11b^+$ c-DC respond to all known TLR ligands. Murine Flt3-ligand derived pDC ($CD11b^-/CD11c^+/B220^+/CD45RA^+$), like their *ex-vivo* counterparts, respond only to TLR7 or TLR9 ligands in short time incubations. There is, however, the caveat not to overemphasize transcriptional expression levels of TLRs by DC subsets. First, mRNA expression does not necessarily correspond with functional protein. For example, high levels of mRNA for TLR8 are expressed in murine DC, but functionally murine DC are completely dependent on

the expression of TLR7 (demonstrated by DC from TLR7 KO mice or the $CD8^+$ DC lacking TLR7 expression) in order to respond to resiquimod, a ligand for TLR7 and human TLR8 [10, 11]. Secondly, TLR expression is modulated by cytokines. Thus a TLR panel defined in resting cells might not reflect the situation after cytokine priming or cell activation. For example, we found that sorted FL culture derived pDC start to respond to TLR2 and TLR3 ligation upon extended (>24 h) culture periods [27].

7.4
TLR Signaling

Signaling via TLR expressed by DC results in an array of activation events including phenotypic and morphological activation, cytokine production and migration. A central adaptor molecule for TLR signaling is myeloid differentation factor 88 (MyD88) as demonstrated by the inability of MyD88 deficient DC to produce inflammatory cytokines (e.g. IL-6, IL-12 or TNF-a) in response to TLR ligation [30]. Upon TLR triggering MyD88 becomes recruited by homophile TIR interactions to the corresponding TLRs. As a consequence, IL-1 receptor-associated kinase (IRAK) 4 becomes recruited, which binds to IRAK1 and forms a complex with the tumor necrosis factor receptor associated factor 6 (TRAF6). IRAK1 and TRAF6 dissociate from the TLR complex and TRAF6 then forms a complex with the TGF-β activated kinase 1 (TAK1) and the TAK1 binding molecules TAB1 and TAB2. This leads to the activation of TAK1 which in turn results in activation of two signal transduction pathways: the NF-κB pathway and the mitogen-activated protein (MAP) kinase pathway which includes p38, JNK1/2 and ERK1/2 [31, 32].

Members of TLR9 and TLR2 subfamilies as well as TLR5 fully depend on the presence of MyD88 for signaling. For MyD88 dependent signaling of TLR2 and TLR4, MyD88 hetero-dimerizes with the adaptor molecule TIR domain-containing adaptor protein (TIRAP also named Mal).

For signaling via TLR3 and TLR4 an additional signal pathway comes into play which is independent of MyD88. The signals via this pathway are mediated by the adaptor molecule TIR domain-containing adaptor inducing IFN-beta (TRIF, also named TICAM). Signaling via this pathway induces delayed activation of NF-κB and IFN regulatory factor (IRF)-3 that results in MyD88 independent DC maturation and induction of IFN-β respectively. The activation of NF-κB is initiated by either activation of TRAF6 or receptor interacting protein (RIP) 1 upon binding to TRIF [33, 34]. The pathway leading to the activation of IRF-3 depends on the non-canonical IkappaB kinase homologs, IkappaB kinase-epsilon (IKKε also named IKK*i*) and TANK-binding kinase-1 (TBK1) [35, 36].

In the case of TRIF dependent signals via TLR4 (but not via TLR3) an additional adaptor molecule named TIR domain-containing adaptor (TRAM, also named TICAM-2) is operative [37, 38].

As mentioned all signaling events via activation of the TLR9 family and culminating in type I IFN (IFN-I) production, DC maturation and the production of in-

flammatory cytokines strictly rely on MyD88. Until recently, the mechanism of production of IFN-I in response to TLR7 or TLR9 ligands was elusive. In general, members of the IRF transcription factor family are thought to be essential in the production of IFN-I. After an initial NF-κB and IRF-3 dependent induction of IFN-β and IFN-α4 (which signal via the IFN-I receptors through the JAK-STAT pathway) high levels of IRF-7 are induced [39]. Activated (phosphorylated) IRF-7 then translocates into the nucleus and initiates the transcription of the multiple IFN-α genes. An important piece of the TLR signaling puzzle was added recently by the demonstration that IRF-7 and potentially also IRF-5 directly interact with MyD88, thus explaining the interferonic potential of TLR7 and TLR9 ligands [40–42]. Furthermore, the high IFN-α production by pDC (but not other cells) in response to ssRNA or CpG-DNA can be explained by the constitutive high levels of IRF-7 in pDC [43].

7.5 What Determines the Outcome of the Immune Responses?

Since many aspects of DC activation including cytokine production, migration, antigen processing and presentation are dealt with in detail in the corresponding chapters of this book, we will focus here on the following question: Does the nature of the DC subset or the quality of the danger signal perceived by TLRs determine the fate of the immune response (e.g. TH1 vs TH2, tolerance versus immunity, antibody responses versus CTL induction)?

DC are key in inducing adaptive immune responses. In particular, their outstanding ability to stimulate naïve T cells characterizes them as professional antigen presenting cells (APCs). Additionally, DC directly or indirectly interact with other immune cells such as NK cells or B cells and thus display diverging functions that allows shaping of immune responses.

In the periphery, and to some extent in the lymphoid organs, DC are found to be immature [44]. Immature DC continuously sample their environment for antigens and are thought to be involved in maintaining or inducing tolerance since they anergize/clonally delete Ag-reactive T cells. Upon exposure to endogenous stimuli (e.g. cytokines or CD40-Ligand) or exogenous stimuli (e.g. TLR ligands) DC maturation is triggered. Upon activation DC start to produce a variety of cytokines and chemokines which recruit and activate cells of the innate immune systems (e.g. NK cells, neutrophils, monocytes) that are able to contain or at least retard an ensuing infection. Furthermore DC maturation is accompanied by dramatic changes in antigen uptake and processing, cell morphology, phenotype and migratory patterns. The environment sampling is arrested in the maturing DC and the cells migrate to the draining lymph nodes. MHC molecules and costimulatory molecules (e.g. CD80, CD86) are upregulated and the processed antigens will be presented to MHC class II restricted T helper and to MHC class I restricted cytotoxic T cells (crosspresentation).

If the DC subset that first encounters a pathogen were responsible for directing the immune response then hardwiring of that particular DC must be critical. The hardwiring could include PAMP recognition molecules that are expressed, signaling pathways that are utilized and even the localization or 'residency' of particular DC subsets. The restricted TLR expression pattern of particular DC subsets may confer "blindness" to the corresponding TLR ligand. For example, murine $CD8^{pos}$ splenic DC encountering a TLR7 ligand or pDC encountering LPS or human cDC encountering CpG would be nonresponsive [29, 45]. Likewise differences in expression of certain transcription factors entail different DC subsets with a huge variation in cytokine production upon TLR stimulation. For example, the murine $CD8^{pos}$ splenic DC subset expresses high levels of IRF1 and IRF8 and these factors are likely to mediate the high IL-12 production by this DC subset in response to TLR ligation [46]. The high constitutive expression of IRF-7 in pDC is likely to enable them to produce extraordinarily high levels of IFN-I in response to ligation of members of the TLR9 subfamily [43].

However nearly all forms of the hardwired DC subset are subject to positive and negative signals that are certain to augment the complexity of immune networks. Inhibitory signals encountered during or before challenge with a TLR ligand may modify the DC subset specific response, an example being the inhibition of IL-12 producing capacity of DC by prostaglandins [47]. This type of inhibitory mechanism may even happen without any benefit for a pathogen as recently shown for pollen derived phytoprostanes that inhibit IL-12 production [48]. On the other hand numerous examples for positive changes or priming of DC hardwiring exist, for example the positive feedback loops of cytokines such as IL-4 or IFN-γ that enhance the IL-12 producing capacity of DC [49].

Indeed the "danger signal" itself influences the nature of immune responses. TLR-2 ligation induces low levels of IL-12 but high levels of IL-10 whereas TLR9 ligation induces the reverse, leading to TH2 or TH1 responses respectively [50, 51]. Moreover, the activation of DC via TLR mediated pathogen recognition is, as expected, subject to pathogen encoded strategies to interfere with this activation. *Vaccinia* virus and *Hepatitis* virus are just two examples of pathogens that encode proteins able to interfere with TLR dependent signaling, thus evading consequences of their recognition [52, 53]. Even more sophisticated is the situation described for *Yersinia* shown to produce a TLR2 binding protein able to cause immunosuppression by triggering IL-10 production [54].

The discovery of the TLR system as a major pathogen sensing mechanism employed by a variety of nonimmune and immune cells such as DC, allowed us to fill some gaps in our understanding of how immune responses become initiated and directed. The fate of an immune response upon pathogen encounter is a complex, interwoven result of specific DC function and pathogen characteristics. At the intersection of the innate and the adaptive immune systems two important and highly connected principles of immune recognition stand out: the TLR as molecular and the DC as cellular sensors.

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8
C-type Lectins on Dendritic Cells: Antigen Receptors and Modulators of Immune Responses

Yvette van Kooyk and Teunis B.H. Geijtenbeek

8.1
Introduction

The initial molecular interaction of pathogens with DCs is crucial in determining the type of effector T cell that differentiates to induce an immune response. In particular, pattern recognition receptors (PRR) such as C-type lectins (CLRs) and Toll-like receptors (TLRs) have an important role in the recognition and internalization of pathogens. CLRs are particularly important for recognition and internalization of glycosylated antigens, which are subsequently processed and presented on MHC class I and II molecules. CLRs are for this reason also called endocytic receptors [1, 2]. TLRs are key elements for the induction of intracellular signaling cascades after recognition of pathogenic components; TLR ligation induces DC maturation and activation. Some pathogens have evolved several strategies to evade anti-pathogenic responses of the host by subverting the function of these pattern-recognition receptors. Recent examples of pathogens that exploit the intracellular routing of carbohydrate-recognizing CLRs to escape antigen presentation, or that escape immune activation through interference of the crosstalk between CLR and TLR signaling, will be discussed [3–5]. On the other hand specific targeting of antigens to CLRs may facilitate the antigen presentation capacity of DCs or may induce tolerance to suppress inflammatory responses and may be beneficial in auto-immunity. Alternatively, targeting of antigens to CLRs in combination with a TLR agonist may induce and enhance immunogenic DC mediated responses, beneficial for anti-tumor responses in cancer. Thus, the balance between TLR and CLR activation on DC upon antigen recognition may be instrumental in inducing either tolerance or immune activation.

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8.2
DCs and Antigen Recognition Receptors

Immature DCs are well positioned throughout the body to sense and capture invading pathogens for efficient antigen presentation to naïve T cells. DCs have the capacity to direct the differentiation of T helper cells into distinct effector cell sub populations. These include Type 1 (Th1) and Th2 effector T cells against intracellular and extracellular pathogens, respectively, and regulatory T cells (Treg) which sustain tolerance against self-antigens and thus dampen excessive immune responses to pathogens [6].

Especially immature DCs express an abundant variety of PRRs to interact with invading pathogens that recognize characteristic molecular patterns within microbial carbohydrates, lipids and nucleic acids [7]. Such receptors include the Toll-like receptors (TLRs) [8, 9] and the C-type lectins (CLRs) [10]. TLRs relay information from the interacting pathogen to DCs through intracellular signaling cascades, thereby eliciting appropriate cellular processes, such as DC maturation and/or the induction of pro-inflammatory cytokines (IL-12, IFNγ) [7, 8]. In contrast, CLRs recognize carbohydrate structures on antigens and internalize antigens for antigen processing and presentation, without induction of DC maturation [2, 11]. Thus far more than 15 CLRs have been identified on DCs and macrophages [5]. Although both TLRs and CLRs recognize different determinants and have distinct functions, recent studies suggest that CLRs may also modulate immune reactions through crosstalk with other receptors and especially with TLRs [3, 4, 12, 13]. This indicates that the outcome of the immune response is determined on the balance between triggering of the two receptor families.

8.3
CLRs as Antigen Receptors for Homeostatic Control

Most CLRs function as antigen receptors that are involved in antigen capture and presentation [11]. Endocytosis by CLRs is guided by their intracellular internalization motifs, whereas some CLRs contain ITIM- or ITAM-like motifs in their cytoplasmic domains, illustrating potential immuno-suppressive or -activating functions of these receptors [2]. CLRs possess different numbers of carbohydrate recognition domains (CRDs) ranging between a single domain (e.g. DC-SIGN, Dectin, MGL) and eight to ten different CRDs (e.g. MR and DEC205). Some CLRs recognize N-linked glycosylations, whereas others interact specifically with O-linked glycans, but the exact specificity of the most CLRs for their glycan ligands remains to be determined. While some CLRs recognize monosaccharides, such as mannose, fucose or galactose, others recognize more complex sugar moieties as expressed by glycoproteins and glycolipids. The specificity of CLRs appears to be determined by multimerization of the receptor, the branching of the carbohydrates and the protein backbone of the glycoprotein that exposes the carbohydrate structure [1] (see

http://web.mit.edu/glycomics/consortium/). *O*-linked structures are often exposed on collagens, mucins and some pathogens, whereas *N*-linked structures are present on the vast majority of glycoproteins in the body as well as on pathogens that use the host's glycosylation for their survival and spread, such as retroviruses [5].

Several pathogens that target the CLRs DC-SIGN, MR and Dectin-1 seem to subvert the function of these CLRs [4, 5] either by inhibition of antigen presentation or modification of T cell responses. The physiological function of CLRs may be recognition of glycosylated self-antigens for homeostasis control [14, 15]. The *in vivo* localization of CLRs on immature DCs in peripheral tissues supports an important function of CLRs in the clearance of self-antigen and tolerance induction. DC-SIGN and the MR are highly expressed by DCs in placenta at the interface of mother/child antigen transmission, a site where the maintenance of immune tolerance plays a central role [16]. Indeed, both *in vitro* and *in vivo* antigen-targeting to CLRs on immature DCs leads to tolerance, suggesting that tolerance is maintained by immature DCs that have captured self-glycoproteins through CLRs and this leads to the induction of Treg [17]. Thus, the continuous interaction of carbohydrate determinants on self-glycoproteins with CLRs on resident APC may be important for homeostatic control.

8.4 CLRs as Adhesion Receptors

There are several indications that CLRs may function as adhesion receptors [5]. In particular, the DC-specific CLR DC-SIGN functions as an adhesion receptor that mediates cellular interactions of DC with endothelial cells through ICAM-2 [18] and with T cells through ICAM-3 [19] probably by recognizing high-mannose moieties on these counterstructures (Fig. 8.1). Recently, Mac-1 was identified as a third cellular ligand for DC-SIGN that allows neutrophil interactions with DCs, thereby modulating DC function [20]. In contrast to its other cellular ligands, DC-SIGN interacts with Lewis antigens on the CD11b chain of Mac-1. DC-SIGN interacts with both mannose- and fucose-containing carbohydrates, such as high mannose structures and lewis antigens, respectively [21, 22]. Strikingly, even though Mac-1 is expressed by various cell-subsets including macrophages and DCs, only neutrophils were found to express Mac-1 with Lewis antigens [20]. Glycosylation is a cell-specific process that depends on the expression levels of many glycosyltransferases but also on the protein backbone of the glycoprotein. Further research is necessary to determine the neutrophil-specific glycosylation of Mac-1. Interestingly, on neutrophils neither ICAM-2 nor ICAM-3 express Lewis antigens and these structures were not involved in DC-SIGN binding [20]. This strongly indicates that, besides specific proteins, also cell-specific glycosylation may determine the interaction with CLRs on DCs.

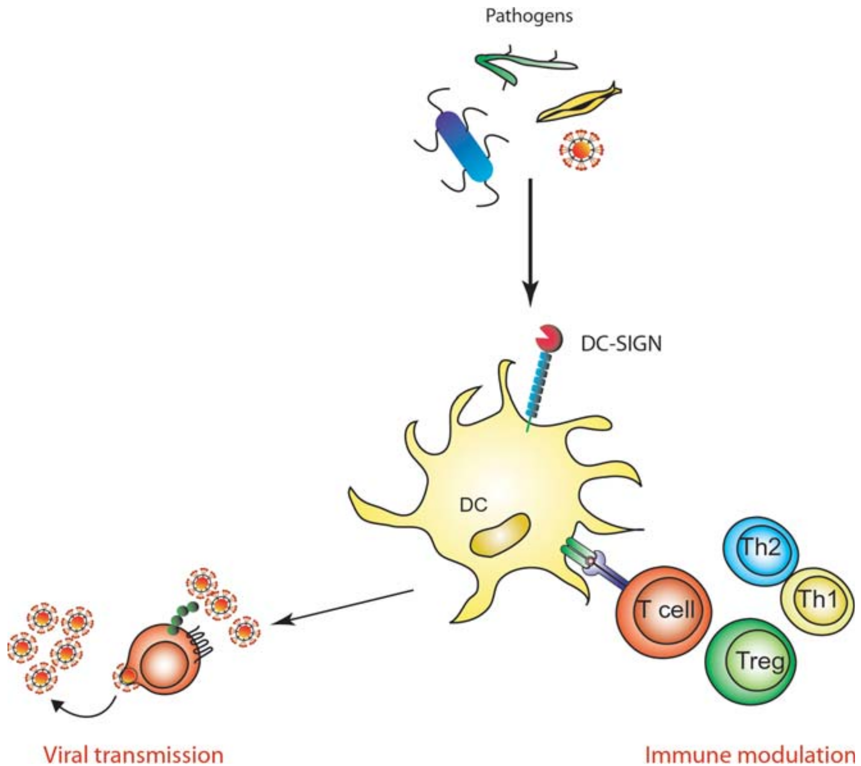

Fig. 8.1 The initial interaction of pathogens with DCs determines the immunological outcome. CLRs such as the C-type lectin DC-SIGN recognizes different pathogens through their carbohydrate structures. Although many pathogens interact with this receptor, it is becoming evident that the immunological outcome of this interaction is specific for the pathogen. DC-SIGN might cooperate with other innate immune receptors, such as Toll-like receptors to fine-tune the immune responses.

Neutrophils are key players of the innate immune system that provide a first line of defense against invading pathogens. The contribution of neutrophils in adaptive immune responses is not completely clear yet. Van Gisbergen et al. [20] demonstrated that neutrophils can imprint their information on DCs to modulate adaptive immune responses. Activated neutrophils strongly clustered with immature DC, which resulted in DC maturation and IL-12 production. Co-culturing these DCs with naïve T cells demonstrated that the DCs induced a strong Th1 response. Thus, DC-SIGN and Mac-1 define a novel molecular pathway to establish cellular adhesion between DC and neutrophils providing a novel cellular link between innate and adaptive immunity (Fig. 8.2).

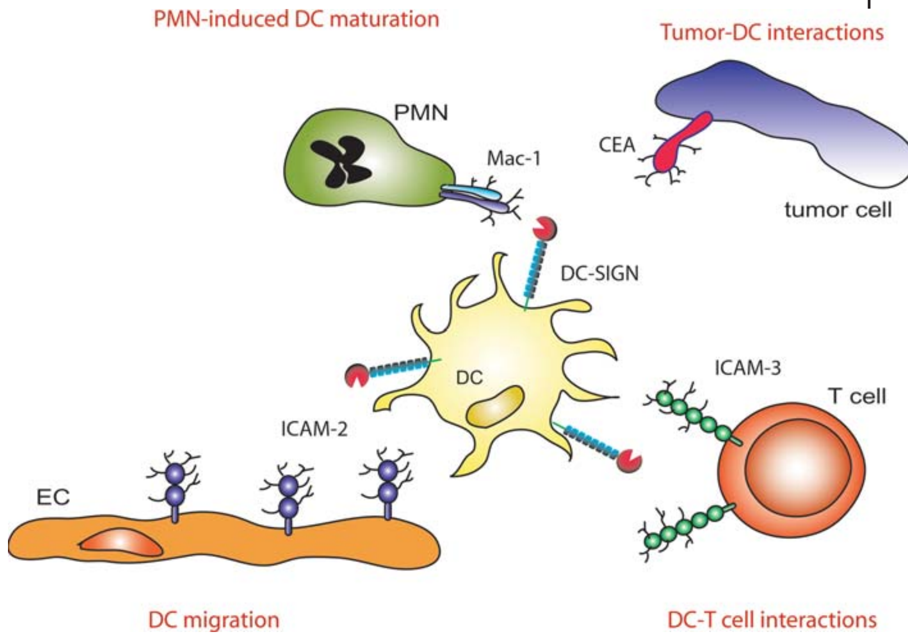

Fig. 8.2 C-type lectins govern cellular communications that may modulate adaptive immune responses. CLRs such as the C-type lectin DC-SIGN functions as an adhesion receptor by facilitating Cell-cell interactions upon recognition of glycan structures on self glycoproteins. DC-SIGN facilitates trans-endothelial migration of DCs through ICAM-2 and initiates DC-T cell interactions through recognition of ICAM-3 which allow scanning of the presented MHC-peptides complexes by T cells. Activated neutrophils strongly cluster with DCs, which modulates DC-induced immune responses, thus providing a link between innate and adaptive immune responses. Upon malignant transformation, colon epithelial cells change their glycosylation, resulting in an altered glycosylation of tumor-specific antigens such as CEA. Tumor-derived CEA, but not normal CEA, is recognized by DC-SIGN on DCs, which might result in protective immune responses or allows unbridled growth of the tumor.

8.5 CLRs as Pathogen Receptors

8.5.1 HIV-1 as Prototypic Example for Virus-DC-SIGN Interactions

Most CLRs on DC have internalization motifs in their cytoplasmic domain, and internalize glycosylated antigens upon binding. DC-SIGN is a DC-specific CLR with affinity for both high mannose structures and Lewis antigens [1, 19, 21]. Ligand binding by mulitmers of DC-SIGN that are concentrated on the surface of DCs [23, 24] results in rapid internalization from the cell surface, and the ligand-DC-SIGN complexes are targeted to late endosomes/lysosomes [11]. Thus, DC-SIGN, similar to other CLRs such as Langerin, MR, DEC205 and Dectin-1, func-

tions as an antigen receptor. One of the first pathogens identified to interact with DC-SIGN was the HIV-1 envelope glycoprotein gp120 that binds DC-SIGN with high affinity [25, 26]. Although DC-SIGN has the capacity to internalize antigens for loading on MHC class II [11], the interaction with HIV-1 does not necessarily lead to antigen presentation. Instead DC-SIGN acts as a HIV-1 trans-receptor that mediates capture of HIV-1 by DCs at sites of entry and efficiently transmits the virus to target T cells [26]. Upon internalization, virus particles are evenly distributed inside DCs and within minutes after the initial DC-T cell contact they concentrate at the T-cell-DC interface, the so-called infectious synapse [27]. The formation of these infectious synapses may increase the local concentration of virus particles, and facilitate efficient transmission to T cells [27]. Only a minor part of the internalized virus may be protected [28] and the majority of viral particles are degraded for antigen presentation [29], suggesting that different sorting events may take place simultaneously. Although several studies indicate that HIV-1 is protected by DCs, the extent of protection is not yet fully understood. Recently, it was shown that the half-life of captured virus in DCs may be shorter than expected and that long-term carriage of virus is via infection of DCs at low levels [30].

Immature DC-SIGN$^+$ DCs are located beneath the mucosal epithelium likely mediate HIV-1 transmission. Although immature DCs express several C-type lectins, antibody blocking studies and RNA interference experiments have demonstrated that DC-SIGN is the primary receptor for HIV-1 on these cells [26, 31]. Strikingly, disruption of DC-SIGN expression in immature DCs through lentiviral-mediated RNA interference impaired infectious synapse formation between DC and CD4$^+$ T cells, since virus was not transported to the DC-T cell interface [31]. Further research will be needed to fully understand the mode of protection, transmission and formation of infectious synapse.

Besides HIV-1, a wide range of other viruses are known to interact with DCs via DC-SIGN. DC-SIGN also binds and internalizes Dengue virus, HCMV, HCV, Ebola and SARS virus for in-*trans* infection of target cells [32-36]. In contrast to HIV-1, HCMV, Dengue and Ebola virus directly infect DC through interactions with DC-SIGN [32, 33, 35].

In summary, data strongly suggests that a range of viruses target DC-SIGN on DCs for transmission to target cells in a similar fashion as described for HIV-1. Preventing the interaction with DC-SIGN or disrupting the infectious synapse formation may be a powerful strategy to intervene these infectious diseases.

8.6
Glycan Modifications and Pathogen Recognition by DCs

A detailed study of the carbohydrate specificity of DC-SIGN revealed its affinity for many pathogens that express mannose-containing carbohydrates or Lewis antigens [21, 37] including *Mycobacteria tuberculosis* [13, 38], *Helicobacter pylori* [21], *Schistosoma mansoni* [39] and Leishmania [40]. Also other CLRs such as the MR may recognize several mannose-exposing pathogens such as HIV-1 [41] and *Can-*

dida albicans. In contrast, also CLRs with a GalNAc specificity such as MGL binds pathogens such as *S. mansoni* antigens [42] and filoviruses [43].

A major component of the cell wall of *M. tuberculosis* is mannosylated lipoarabinomannan (ManLAM), a mannose-containing glycolipid that has been implicated as both a virulence factor and a stimulus of host defense mechanisms [44]. The LAM from pathogenic *M. tuberculosis* strains is between 40–70% mannose-capped, whereas the fast growing *M. Smegmatis* contains only uncapped AraLAM [44]. Most slow growing virulent mycobacteria strains contain the exterior mannose-cap of ManLAM that interacts with DC-SIGN, whereas most avirulent mycobacteria strains lack the mannose cap. Recent data demonstrate that ManLAM inhibits LPS-induced DC maturation by occupying the CLR DC-SIGN. Inhibitory anti-DC-SIGN antibodies fully restore the LPS-induced DC maturation in the presence of ManLAM [13]. Also viable *M. bovis* BCG induces DC maturation [13], probably through TLR-2 and TLR-4 signaling and ManLAM binding to this CLR inhibits the maturation of DC demonstrating its suppressive effects on DC [45]. ManLAM binding to DC-SIGN also induces the production of anti-inflammatory cytokines by LPS-activated DCs [13]. This indicates that the CLR DC-SIGN upon binding ManLAM, may deliver a signal that interferes with TLR-4-mediated activation.

The inhibition of DC maturation and the induction of IL-10 by secretion of glycosylated products of pathogens, such as ManLAM, may contribute to the virulence of mycobacteria; immature DCs and IL-10-treated DCs are not only less efficient at stimulating T cell responses but also induce a state of antigen-specific tolerance [46]. It is interesting to see that pathogens, such as mycobacteria, have evolved a survival strategy within the host environment, by altering their glycosylated cell-wall components to occupy CLRs, such as DC-SIGN, to silence immune activation.

Also other pathogens interact with immature DCs through DC-SIGN by changing their glycosylation, which results in modulation of immune responses. For example, *H. pylori* lipopolysaccharide (LPS) expresses Lewis antigens that are subject to phase variation [47]. Frameshifts in glycosyltransferase genes results in on and off switching Lewis antigens expression on the bacterial LPS structures. Strikingly, these modifications dictate a differential targeting of DCs; the *H. pylori* expressing Lewis-containing LPS strongly binds DC-SIGN on DCs, whereas the Lewis-negative LPS does not interact with DC-SIGN [48]. DCs expressing DC-SIGN are abundantly present in the lamina propria of the stomach, suggesting that DC-SIGN may facilitate capture of *H. pylori* from the stomach lumen. A more in-depth study of the consequences of this interaction revealed that the Lewis$^+$ phase variant induced IL-10 expression by DCs, which was inhibited by antibodies against DC-SIGN [48], suggesting that this variant modulates immune responses through its interaction with DC-SIGN. Moreover, the Lewis$^-$ *H. pylori* triggered Th1 responses, whereas Lewis$^+$ *H. pylori* through interactions with DC-SIGN blocked Th1 induction and shifted the immune response towards Th2 [48]. This mechanism might enable the few Lewis$^+$ *H. pylori* in a predominant Lewis$^-$ population to suppress protective Th1 responses, and provide an explanation for the induction of chronic gastritis by *H. pylori* infection. Interestingly, the Lewis$^+$ and Lewis$^-$ strains are

identical apart from the Lewis antigen expression on the LPS, suggesting that the interaction with DC-SIGN enables the Lewis$^+$ strain to modulate DC function. Pathogens such as *H. pylori* may benefit form glycan modification that cause modulation of DC function through targeting of CLRs.

Also, certain developmental forms of the worm *S. mansoni* target DC-SIGN. Soluble antigens, especially glycans, released by eggs elicit strong immune responses leading to granuloma formation. Several of these soluble egg antigens (SEA) are bound by DC-SIGN via Lewis x [39]. Recent work shows that a specific lipid from Schistosoma eggs and worms modulates DC function resulting in Treg development via TLR2, probably in combination with an unidentified CLR receptor [49].

Recently, we found that various strains of Lactobacilli, one of the frequently used probiotics that suppress local immune activation in the intestine, target DC-SIGN to induce Tregs (H. Smits, personal communications), Glycan structures present on these immune suppressive microorganisms that particular target the CLR DC-SIGN, are currently evaluated.

8.7 CLRs as Signaling Receptors

In addition to ManLAM, several recent studies have demonstrated that crosstalk between C-type lectins and TLRs can occur either in a synergistic or antagonistic fashion [3, 4, 12]. Dectin-1, a yeast binding C type lectin was demonstrated to synergize with TLR-2 to induce TNFα and IL-12 [4]. Recently it was shown that Dectin-1 can also promote synthesis of IL-2 and IL-10 through phosphorylation of the membrane proximal tyrosine in the cytoplasmic domain and recruitment of Syk kinase [50].

In a recent study, targeting of the MR was demonstrated to induce activation of DCs leading to the production of anti-inflammatory cytokines and the induction of Th2 cells with regulatory capacity [51]. MR triggering could also antagonize the induction of inflammatory cytokines by the TLR-4-ligand LPS, implying crosstalk between the MR and TLR4 leading to downmodulation of TLR-4 signaling. Strikingly, besides an activating anti-MR antibody, only certain natural ligands for the MR such as ManLAM could prime regulatory DCs, whereas others, such as mannan and dextran, had no effects on DCs [51]. This differential effect could be due to the fact that several of these ligands can also bind to other CLRs, including DC-SIGN, and might thus affect activation of DCs.

Similarly an elegant study demonstrated that in-vivo targeting of CLR in the mouse can induce antigen specific auto-immunity when the antigen was coupled to an anti-CLR (DEC-205) specific antibody [17]. Immune activation was achieved by providing simultaneously a strong maturation such as a TLR activator or cross-linking of co-stimulatory molecules.

Thus, the balance between CLR and TLR signaling is instrumental for the outcome of the immune response and may lead to the induction of tolerance or immune activation.

8.8 CLRs and Recognition of Cancer

In particular, the activation and antigen presentation by DCs plays a key role in the initiation of anti-tumor responses. However, tumor cells have found several strategies to dampen antigen presentation and T cell responses initiated by DCs. Tumor-associated antigen (TAA)-interacting with DCs may modify DC function such as production of immune suppressive cytokines and inhibition of DC migration to tumor sites.

TAAs are often auto-antigens that become deregulated, and glycosylation is one of the features that changes during onco-transformation. Such TAA are for example the carcinoma embryonic antigen (CEA) and MUC1 [52, 53]. Both antigens are expressed on cells of normal colon mucosa and epithelial cells, however during onco-transformation, aberrant glycosylation of CEA is a common phenomenon that accompanies colon carcinoma progression [54-56]. These changes include increased expression of Lewis blood group family of antigens, particularly Le^x, and Le^y, that are often linked to disease prognosis. How these posttranslational modifications contribute to tumor cell dissemination and disease severity is not fully understood. Strikingly new findings demonstrate that DCs recognize these modified glycosylations on CEA or MUC1 through CLRs such as DC-SIGN and MGL, respectively, while these CLRs do not interact with ‘normal’ CEA or MUC from colon tissue [57] (S. Saeland, personal communications). Strikingly, tumor-associated CEA from 5 adenocarcinoma cell lines as well as from colorectal cancer patients contains Le^X and Le^Y antigens, and our recent findings demonstrate that in particular the C-type lectin receptor (CLR) DC-SIGN is involved in the recognition of tumor associated CEA with DCs [57]. In situ within the tumor immature DC-SIGN-positive DC are localized but not mature DC, indicating an immune silenced environment [51]. The fact that TAA such as CEA are secreted during metastasis indicates that secretion of modified glycosylated CEA that targets DC-SIGN can lead to systemic tolerance in colon cancer patients. Indeed initial experiments demonstrate that CEA-containing serum from breast carcinoma patients reduces IL-10 and IL-12 production by DCs and DC-induced T cell polarization.

The fact that CLR are highly specific internalization receptors that facilitate and enhance antigen loading on MHC class I and II initiates research to investigate whether they may be useful to optimally load DC with antigens, which may subsequently be used in DC vaccinations to enhance anti-tumor immune responses. Targeting of TAA to DCs in combination with a strong DC maturation stimulus such as a TLR activator may be one of the strategies to *in-vivo* vaccinate DCs with specific CLR targeting TAA and combat tumors.

8.9 Concluding Remarks

Although the function of many CLRs remains unknown, it is becoming clear that CLRs have a key function in the maintenance of homeostasis. Identification of the

self-ligands and the specific carbohydrate structures will be essential to understand the cellular function of many CLRs. The scientific challenge is evident from the fact that glycosylation is not only a highly complex process that involves more than 100 glycosyltransferases and glycosidases, but also a dynamic process that varies in space and time; glycan structures change in association with cellular metabolism and/or differentiation induced by cytokines, hormones, stress and ageing [58].

Lessons of the function of CLRs can be learned from studying pathogens or tumor antigens that specifically target CLRs to evade immune surveillance. Recent studies have demonstrated that internalization pathways of CLRs may depend on the specific ligand, since viruses subvert the internalization pathway to escape degradation and promote infection of target cells. Further evidence was generated from pathogens that target CLRs to interfere with TLR signaling and modulate immune responses to promote their survival. These studies have demonstrated that although both TLRs and CLRs have distinct functions the balance between both receptors upon ligand binding determines the immunological outcome.

Abbreviations

CLR	C-type lectin receptor
DC	dendritic cells
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrin
HCV	hepatitis C virus
LPS	lipopolysaccharide
ManLAM	mannosylated lipoarabinomannan
MR	mannose receptor
PRR	pattern recognition receptors
SEA	soluble egg antigen
Th1	T-helper 1 cells
Th2	T-helper 2 cells
TLR	Toll-like receptor
Treg	regulatory T cells

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9
Scavenger Receptors on Dendritic Cells

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9.1
Introduction

Myeloid cells have mastered the use of a wide range of receptors, termed pattern recognition receptors, to recognize endogenous as well as microbe-associated molecules in order to sample their environment. In macrophages (Mϕ) this leads to maintenance of tissue homeostasis through efficient elimination of harmful metabolites and apoptotic cells and containment of infectious agents and elicitation of inflammatory responses. In dendritic cells (DC) these receptors are exploited to maximize the range of antigens (Ag) presented to the acquired immune system, and to condition the phenotype of the cell according to the challenge it has been exposed to. Therefore, acquired immunity is tailored through the combined effort of pattern recognition receptors.

Microbial recognition is mostly mediated by Toll-like receptors (TLR) and NOD-like proteins, but it is becoming increasingly clear that this recognition can be modulated through engagement of other receptors such as C-type lectins and scavenger receptors (SR) which have previously been associated with endogenous clearance.

The concept of SR was first proposed by Brown and Goldstein when investigating the formation of lipid-laden foam cells in atherosclerotic plaques. These authors proposed this functional definition for receptors able to recognize modified low density lipoprotein (mLDL), such as oxidized or acetylated LDL, but not native LDL. The range of ligands recognized by these molecules has expanded and includes a wide selection of polyanionic structures of endogenous as well as microbial origin. Therefore while most research into SR has concentrated on their contribution to Mϕ and endothelial biology in relation to atherosclerosis, increased interest in their contribution to innate immunity, in general, and to DC biology, in particular, has been generated.

Krieger et al. classified SR into six classes (A, B, C, D, E and F) based on their domain structure which is heterogeneous and includes trimeric proteins with collag-

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enous and coiled coil regions, proteins with lectin domains and proteins with EGF repeats.

9.2
Structure and Binding Properties of Mammalian Scavenger Receptors

In this chapter we will focus only on SR for which functions relevant to DC biology have been described, regardless of the lack of information concerning their expression on DC (Fig. 9.1). This is a growing research area and experience teaches us that resourceful DC can use a wide range of receptors, sometimes in unexpected ways, to enhance their Ag internalization capacity and to tune the activation of the acquired immune system to the immunological challenge. Therefore, even though a systematic analysis of the distribution of SR receptors in DC *in vitro* and *in vivo* is lacking, it is likely that favored by the inherent versatility and heterogeneity of myeloid cells, many SR could play a role in DC biology *in vivo*. For example it is well-established that the range of receptors expressed by DC, as well as the population(s) of DC present in a particular anatomical location, is altered upon stimulation. The recent findings by Doyle et al. support these assumptions. These au-

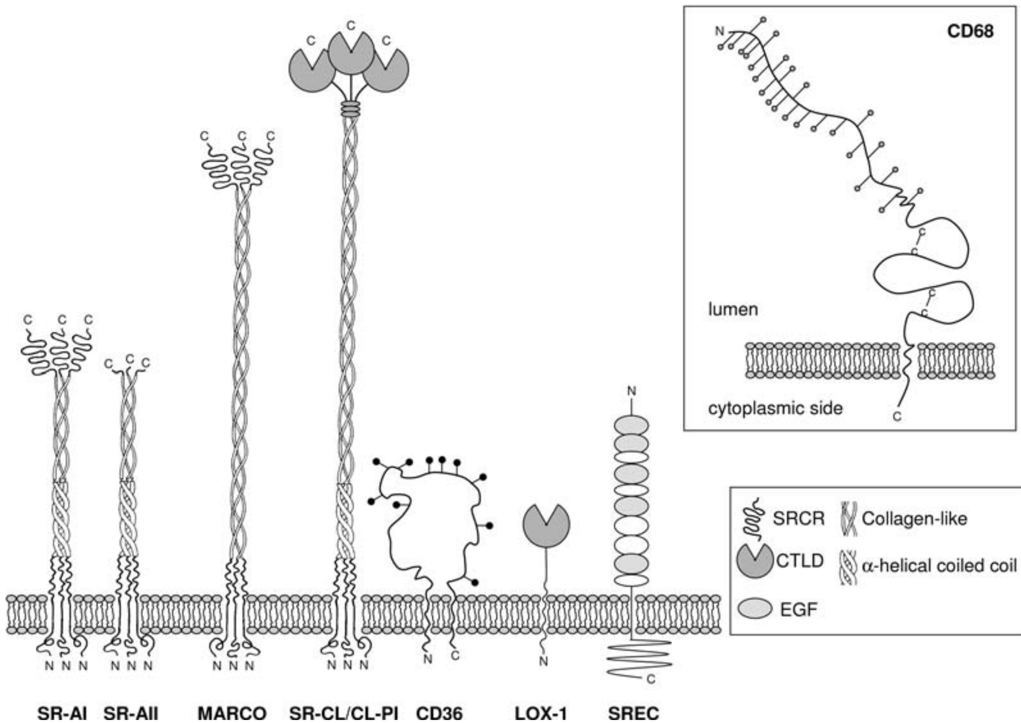

Fig. 9.1 Schematic representation of the members of the SR family described in this chapter.

thors demonstrated increased phagocytic activity in Mϕ treated with TLR agonists. This enhanced internalization activity is restricted to bacteria (it does not affect internalization of latex beads) and correlates with upregulation of several scavenger receptors involved in bacterial recognition [1].

9.2.1 Class A Scavenger Receptors

9.2.1.1 SR-A I, II and III (SR-A)

SR-A I,II and III (SR-A) are generated by alternative splicing of one gene [2]. They are trimeric type II transmembrane glycoproteins with a cytoplasmic tail, a transmembrane region followed at the extracellular portion by a spacer, a α-coiled coil region, a collagenous domain and a C-terminal cysteine-rich domain (scavenger receptor cysteine-rich domain, SRCR) that is absent in SR-A II and III. SRA-III is nonfunctional; it lacks part of the collagenous region and is retained in the endoplasmic reticulum [3]. To date no functional differences have been observed between SR-AI and SR-AII.

The collagenous region has been identified as the binding site for modified lipoproteins [4] while the α-helical coiled coil region is thought to be responsible for receptor trimerization. The cytoplasmic tail mediates endocytosis, phagocytosis and adhesion. Recently Kosswig et al. demonstrated that amino acids in the cytoplasmic tail proximal to the membrane are required for SR-A post-translational processing and trafficking to the cell surface and were sufficient for SR-A-mediated adhesion [5]. Ligand internalization required additional motifs present in the cytoplasmic tail, probably the VXFD motif characterized by Morimoto et al. [6].

In addition to acetylated and oxidized LDL, SR-AI and II bind gram positive [7] and gram negative bacteria [8, 9]. Even though the lipid A component of lipopolysaccharide [10] can be recognized by SR-A, in the case of *Neisseria meninigitidis* a lipid A-independent recognition by SR-A has been demonstrated [9]. In this model SR-A-mediated recognition was not required for TNFα production by Mϕ. SR-A recognizes lipoteichoic acid (LTA) [7] and has different specificities for different LTA molecules [11]. This has been attributed to the distribution of the negative charge on the different LTAs. Zhu et al. demonstrated that while SR-A can bind DNA, it is not essential for the uptake of CpG DNA or its immunostimulatory activity [12].

Mice deficient in SR-A showed enhanced susceptibility to *Listeria monocytogenes* [13] and *Staphylococcus aureus* infection [14] suggesting a protective role for SR-A during bacterial infection. However, there is conflicting evidence regarding the role of SR-A in protection from endotoxic shock. Haworth et al. demonstrated increased susceptibility of Bacillus Calmette–Guerin (BCG)-primed SR-A deficient mice to LPS challenge and this correlated with increased TNFα production. Kobayashi et al. found that levels of interleukin (IL)-1β expression and serum IL-1β were lower in SR-A deficient mice in response to LPS and that these mice were less susceptible to endotoxin shock in response to large doses of LPS. These authors showed that mortality rates could be reduced by pretreatment with an IL-1 receptor antagonist [15].

SR-A can also bind to and phagocytose apoptotic cells [16] though no effect could be observed in SR-A-deficient mice, probably due to receptor redundancy [17]. Berwin et al. have recently demonstrated that SR-A mediates the recognition and internalization of gp96 and calreticulin (CRT) in murine Mϕ and human cells expressing SR-A under an inducible promoter [18]. SR-A is thought to account for approximately 50% of the total endocytic capacity for these proteins. SR-A has also been implicated in cell nibbling from live cells (see below) [19].

SR-A expression has been characterized in detail in the mouse using the mAb 2F8. This receptor has been detected in the red pulp and marginal zone of spleen, thymic medulla and subcapsular region of lymph nodes. Kupffer cells in the liver, alveolar Mϕ in the lung and lamina propria Mϕ in the gut also reacted with 2F8. SR-A was not detected on any non-Mϕ cells, with the exception of sinusoidal endothelial cells in the liver. In the spleen, lymph node and liver, SR-A expression was associated specifically with phagocytic cells which had taken up colloidal carbon [20]. M-CSF increased SR-A synthesis in murine Mϕ. In addition, the Th1 cytokine IFNγ and the Th2 cytokine IL-4 had differential effects on SR-A glycosylation *in vitro* suggesting a further possible regulatory role by these lymphokines on SR-A function [21].

SR-A has been detected in human cultured monocyte-derived DC (moDC) and monkey interdigitating LN DC ($MHCII^{bright}CD83^{bright}$) [19]. Upon maturation of human moDC in response to CD40L a switch from the SR-AII isoform to SR-AI has been observed [19]. Germ-line mutation of SR-A has been associated with increased risk of human prostate cancer and a negative correlation between the infiltration of SR-A expressing APC and prostate cancer progression has been described [22].

9.2.1.2 **MARCO**

MARCO (Mϕ Receptor with a Collagenous Structure) possesses an overall structure similar to that of SR-A I but is produced by a distinct gene. MARCO was first described in the mouse by Elomaa *et al.* [23] and subsequently in man [24, 25]. Akin to other SR, MARCO is an integral membrane protein consisting of an intracellular domain mediating endocytosis and phagocytosis, a membrane spanning region, a short spacer domain followed by the collagenous domain and finally the carboxy-terminal SRCR domain. The collagenous domain of MARCO differs from that of SR-AI as it forms a long continuous region, interrupted at a sole site by an Ala-Gly-Lys sequence. This is thought to form a hinge region in the triple helix. In comparison SR-AI has an extended noncollagenous helix and only a small collagenous domain. MARCO lacks the alpha helical coiled-coil domain. Potential glycosylation sites have been identified in the spacer region and the putative hinge region in the collagenous domain. These are conserved between man and mouse but the functional consequences are not known.

In reported binding studies MARCO binds acetylated LDL, *E.coli* and *S. aureus*, but not the yeast component zymosan or Ficoll, a neutral polysaccharide [23]. Specific binding was confirmed by inhibition with poly G, a well established polyanionic inhibitor of SR ligand binding. Endogenous ligands for this molecule have al-

so been described on B lymphocytes. Karlsson et al. detected a direct interaction between MARCO on marginal zone Mϕ and marginal zone B cells. Activation or disruption of this interaction resulted in marginal zone B cell migration to the follicle. In response to *S. aureus* marginal zone Mϕ moved into the red pulp while marginal zone B cells migrated into the follicular zone [26].

The predominant ligand binding domain of MARCO has been mapped to the arginine-rich segment (residues 432–442) of the SRCR domain with RXR motifs being of particular importance [24, 27]. Some minor bacterial binding activity could also be detected in the collagenous domain [27], but was not investigated further. These results clearly suggest that the ligand-binding properties of MARCO differ from those of other SR-A members, as their ligand-binding function had been located in the collagen domain [4]. However it is also possible that residues 432–442 in the cysteine-rich domain are necessary for correct folding and not binding. This could allow binding to occur at distant sites elsewhere in the receptor. An interaction between uteroglobin-related protein-1, a soluble protein produced by Clara-like cells in the bronchial epithelium, and MARCO has been proposed [28].

Expression of MARCO in cell lines induced formation of large lamellipodia-like structures and of long dendritic processes accompanied by disassembly of actin stress fibers and often also by complete loss of focal adhesions. Expression studies with a variety of truncated MARCO forms indicated that the proximal segment of SRCR is important for this activity [29].

This molecule is expressed on both Mϕ and DC but studies concentrating on its expression on known DC subsets in man or mouse are currently lacking.

In the mouse, MARCO is constitutively expressed by splenic marginal zone Mϕ [23], the medullary Mϕ of lymph nodes [30] and alveolar Mϕ [25]. It is clear that the expression of MARCO is more restricted in comparison with the other SR-A molecules under steady-state conditions. However upon antigenic stimulation with BCG infection, bacterial sepsis or purified LPS *in vivo*, MARCO expression is readily upregulated and induced on red pulp Mϕ and Kupffer cells of liver [30]. Thioglycollate-elicited peritoneal Mϕ also express this receptor. Inhibitory monoclonal antibodies raised against MARCO were used *in vivo* to assess the contribution of this receptor to bacterial clearance. As the overall clearance and killing of both *E. coli* and *S. aureus* was not significantly influenced by antibody treatment and the knowledge that several other Mϕ receptors can also bind and phagocytose microbes, it was concluded that there is redundancy in these bacterial clearance pathways. MARCO expression has been observed in hamster alveolar Mϕ [25].

Treatment of the Mϕ cell line, J774.2, with a panel of pro-inflammatory cytokines including IFNγ, IL-6, TNFα, and IL-1 did not cause any increase in MARCO expression but treatment with LPS did [30]. These results indicate that the upregulation observed *in vitro,* if not also *in vivo,* is a direct result of LPS recognition and is not caused indirectly by subsequent cytokine release. Several consensus sequences for transcription factors have been identified in the promoter region of MARCO supporting the idea that this molecule is transcribed in response to stimuli. Recently, upregulation of MARCO expression has been observed in mouse peritoneal Mϕ in response to *Neisseria meningitidis* [31].

MARCO(–/–) mice displayed an impaired ability to clear pneumococcal infection from the lungs and showed increased pulmonary inflammation and cytokine release, and diminished survival. *In vitro* binding of *Streptococcus pneumoniae* and *in vivo* uptake of unopsonized particles by alveolar Mϕ deficient in MARCO expression were impaired. MARCO(–/–) mice treated with the "inert" environmental particle TiO(2) showed enhanced inflammation and chemokine expression, indicating that MARCO-mediated clearance of inert particles by alveolar Mϕ prevents inflammatory responses [32].

Full analysis of MARCO expression in human tissues during health and disease has been hampered by the lack of specific antibodies but recently a mAb against human MARCO has been described. This Ab stains neonatal human alveolar Mϕ. Using *in situ* hybridization, analysis of MARCO expression has been performed on tissues from sepsis patients. Under these conditions $MARCO^+$ cells were found in thymus, intestine, splenic white pulp, kidney and Kupffer cells of liver. All $MARCO^+$ cells were considered tissue Mϕ [24]. A polyclonal antibody to human MARCO showed strong immunolabeling of human alveolar Mϕ in BAL and within lung tissue specimens [25].

DC can express MARCO on their surface in response to pulsing with tumor lysates [33]. Similarly MARCO expression is transiently induced in the DC cell line DC1, in response to LPS [34]. DC1 cells are a splenic myeloid cell line maintained in the immature state in the presence of granulocyte-macrophage colony stimulating factor. DC1 cells can be fully matured upon treatment with LPS for example. In agreement with previous observations, expression of MARCO in DC1 cells also resulted in formation of large lamellipodia-like structures and long dendritic processes [29, 34] that correlated with increased bacteria binding at the cell surface and reduced phagocytic activity.

9.2.1.3 **SRCL-I**

SRCL-I (Scavenger receptor with C-type lectin) [35]/Collectin placenta 1 (CL-P1) [36], I was cloned independently by two groups from a human placental cDNA library. Ohtani et al. designated it membrane type collectin from placenta due to the presence of a collagenous region and of a C-type lectin domain in the same molecule as it is the case for collectins such as MBL and SP-A [37]. These authors also identified the murine form of this molecule. SRCL-I/CL-P1 is a type II membrane protein. It possesses a short cytoplasmic tail with the internalization signal YKRF, a transmembrane region, a spacer, a coiled-coil domain, followed by a collagenous domain and a C-type lectin domain. Nakamura et al. identified a truncated form of SRCL-I/CL-P1, termed SRCL-II, that lacks the C terminal C-type lectin domain. Functional studies in transfectants have demonstrated binding to both gram positive and gram negative bacteria (*S. aureus* and *E. coli*), yeast and to oxidized LDL but not acetylated LDL which indicates SR function. This binding was inhibited by polyanionic, but not polycationic compounds. Transcripts of SR-CL I were detected in most human tissues by RT-PCR and northern blot analysis but highest levels were detected in human placenta, lung, heart and small intestine. Immunohisto-

chemical analysis in mouse tissues demonstrated that expression was restricted to vascular endothelial cells. The C-type CRD of SRCL-I resembles that of the *N*-acetyl-galactosamine binding molecule HML [38] as they both share the QPD motif required for carbohydrate recognition in HML. Further analysis of soluble C-type lectin domain of SRCL-I/CL-P1 demonstrated binding to glycoconjugates containing GalNAc that could be inhibited by free GalNAc, L-,D-fucose, D-galactose and the carcinoma-associated antigen, Tn antigen [39].

9.2.2 CD36

CD36 belongs to the class B SR family and is highly conserved between the humans and mouse. CD36 is a 53 kDa glycoprotein containing a C-terminal transmembrane region and an uncleaved signal peptide, which is probably a second membrane-spanning domain. The two short cytoplasmic regions (9–13 aa) can be palmitoylated. It has been proposed that the binding properties of CD36 could be regulated through phosphorylation of extracellular threonine 92. Multiple ligands for CD36 have been identified. CD36 binds thrombospondin 1 (TSP-1) that is found in extracellular matrix and platelet α granules through a binding site placed at aa 93–120 that could be modulated by a site placed at aa 139–155. CD36 has been suggested as the mediator of the antiangiogenic activity of TSP-1. Oxidized LDL recognition has been located to the domain located at aa 120–155. mAb against the immunodominant epitope located at 155–183 inhibits all binding properties including recognition of the modifications that occur in membranes of *Plasmodium falciparum*-parasitized erythrocytes and apoptotic cells. Other ligands include anionic phospholipids, such as phosphatidylserine, collagens I and IV and long chain fatty acids [40]. CD36 acts as a translocator of long chain fatty acids and therefore could act as a mediator of energy metabolism. CD36 deficient mice have altered lipid and glucose metabolism [41]. CD36 has been shown to co-localize with caveolin 1 in caveolae [42] where it might play a role in the trafficking of cholesterol. CD36 might act as a receptor by targeting ligands, as a signaling molecule and/or as a regulator of caveolae function, for example CD36 mediates the oxidized LDL-mediated cholesterol depletion in caveolae of endothelial cells [43]. Recently Zeng et al. demonstrated that, in the steady state, CD36 is localized in lipid rafts but not in caveolae, and that binding of oxidized LDL to CD36 leads to endocytosis through a lipid raft pathway that is distinct from the clathrin-mediated or caveolin internalization pathways [44].

CD36 has been implicated in regulation of lipid metabolism and the development of foam cells during atherosclerosis [41, 45]. Unlike SR-A, CD36 binds to minimally oxidized LDL and LDL oxidized through the myeloperoxidase-hydrogen peroxide-nitrite system of phagocytic cells which might have more relevance under physiological conditions [46]. CD36 cooperates with $\alpha_v\beta_3$ integrin in Mϕ [47] and with $\alpha_v\beta_5$ on DC [48] and retinal pigment epithelial cells [49, 50] in recognition of apoptotic cells. Ligands in apoptotic cells include anionic phospholipids [51] but a

bridging role for TSP-1 has also been described [52]. CD36 has been shown to facilitate recognition of selected TLR2 ligands [53] (see below).

CD36 is expressed in microvascular endothelium, adipocytes, skeletal muscle, epithelia of the retina, breast and intestine and smooth muscle cells. In haematopoietic cells it is present in erythrocyte precursors, monocytes/Mϕ and DC, platelets and megakaryocytes [43] and B cells [54]. In the mouse $CD8\alpha^+$ DC have been shown to differentially express CD36 [55]. CD36 has been also detected in human monocyte-derived DC [48, 56].

9.2.3 LOX-1

LOX-1 (lectin-like oxidized LDL-receptor-1) belongs to the class E SR family and is also a member of the NK family of C-type lectin receptors [57]. It was originally identified by Sawamura et al. as a receptor for oxidized LDL [58]. LOX-1 is a 48–50 kDa type II transmembrane glycoprotein (273 aa in the human form) with the following domains: a N-terminal cytoplasmic tail able to mediate ligand internalization, a hydrophobic transmembrane domain, a neck region and a C-type lectin domain. In addition to oxidized LDL this protein recognizes acetylated-LDL (demonstrated in Cos7-LOX-1 transductants [59]), fibronectin [60], bacteria (see below), Hsp70 [61], activated platelets [62], apoptotic cells and aged/oxidized erythrocytes [63]. LOX-1 expressing transfectants specifically recognized $gram^+$ (*S. aureus*), and $gram^-$ bacteria (*E. coli*). This binding could be inhibited by poly-I and anti-LOX-1 mAb. Contribution of LOX-1 to bacterial recognition in cultured bovine aortic endothelial has also been demonstrated [64]. Mutagenesis studies have shown that binding activity (for acetylated-LDL) is mediated by the C-type-like lectin region [59]. Both positively charged and noncharged residues have been shown to be required for ligand recognition indicating that in this protein binding does not seem to be mediated only by the recognition of negatively charged ligands through positively charged residues.

This protein was originally detected in vascular endothelial cells but it has also been found in mature human and mouse Mϕ and in THP-1 cells [65, 66] as well as in human monocyte-derived DC and human peripheral blood myeloid DC ($CD11c^+$) and mouse bone-marrow derived DC [61]. Expression in DC is decreased upon maturation. It is upregulated by inflammatory cytokines and by oxidized LDL *in vitro* and proatherogenic conditions *in vivo* such as hypertension, hyperlipidemia and diabetes mellitus [67]. In endothelium LOX-1 engagement leads to superoxide and cytokine production, inhibition of NO production and enhancement of endothelial adhesiveness for leukocytes [67].

In a model of fatal endotoxin-induced inflammation anti-LOX mAb inhibited leukopenia and rescued the animals. The same authors implicated LOX-1 in leukocyte rolling under inflammatory conditions *in vivo* and showed that a recombinant LOX-1-Fc protein could mediate PMN binding under physiological shear force, but no information about the percentage of apoptotic PMN present in the preparation was presented [67].

9.2.4
SREC-I

SREC-I (scavenger receptor expressed on endothelial cells I), the class F SR, was originally cloned from endothelial cDNA libraries. The cDNA encodes a protein of 830 amino acids with a calculated molecular mass of 85,735 Da (mature peptide). It contains two hydrophobic regions, in the N terminus and the middle of the molecule, which could serve as a signal sequence and a transmembrane domain, respectively. SREC-I is a type I transmembrane protein, with a long C-terminal cytoplasmic domain relative to the N-terminal extracellular domain. The extracellular domain, which is made up of 406 amino acids, contains five epidermal growth factor-like cysteine pattern signatures. The C-terminal cytoplasmic domain is unusually long (391 amino acids) and is composed of a Ser/Pro-rich region followed by a Gly-rich region.

SREC-I binds and mediates degradation of acetylated LDL. Association of acetylated LDL was effectively inhibited by oxidized LDL, malondialdehyde-modified LDL, dextran sulfate, and polyinosinic acid, but not by natural LDL and heparin [68]. In addition to binding modified LDL, SREC-I can mediate the uptake of CRT and gp96 [69](see below). Although originally described in endothelial cells SREC-I has been also detected in Mϕ where its expression is upregulated by LPS [69, 70].

9.2.5
Other Members of the SR Family

9.2.5.1 CD68

CD68 (mouse macrosialin [71]) is a heavily glycosylated type I molecule localized mainly intracellularly with a short and highly conserved cytoplasmic tail. In the intralumenal region two domains separated by a proline-rich hinge region have been observed. The more internal domain I, has similarities with sequences found in lysosomal–associated membrane glycoproteins such as LAMP1 or LEP100, while the most N terminal, domain II, represents a mucin region that is heavily O-glycosylated. Macrosialin is processed from a 42 kDa precursor protein, to a mature product of 85 to 115 kDa. Glycosylation varies among different Mϕ populations and in response to the phagocytic stimulus zymosan [72]. Oxidized LDL has been identified as a putative ligand for CD68 and macrosialin [73] and a role for macrosialin in endocytic uptake is supported by the detection of 10–15% of the protein at the plasma membrane in elicited peritoneal Mϕ with a very rapid internalization rate [74]. The presence of CD68 is suggestive of a highly developed lysosomal compartment characteristic of Mϕ. $CD68^{+}$ cells can be found in the T-cell areas of secondary lymphoid organs but no co-localization with the DC-related marker MHCII has been reported. Interestingly mouse Langerhans cells have been shown to express CD68 together with the Mϕ-differentiation marker F4/80 [75].

9.2.5.2 SR-B1, FEEL-1 and FEEL-2

Other members of the SR family include SR-B1 a phosphatidylserine [76] and LPS receptor that can also bind serum amyloid A protein [77, 78] and the receptors for the advanced glycation end products, FEEL-1 and FEEL-2 [79]. No information regarding the expression of these molecules in APC is available to date but their involvement in the internalization of modified self by DC under specific conditions and in innate immunity should be taken into consideration.

9.3 Role of Scavenger Receptors in Dendritic Cell Biology

In this Section we will focus on the contribution of SR to Ag acquisition by DC (9.3.1) and to modulation of DC phenotype (9.3.2). In the first instance we will discuss the generation of SR ligands to enhance the immunogenicity of proteins (9.3.1.1), the role of SR in the internalization of Ag associated with apoptotic cells (9.3.1.2) and live cells (9.3.1.3) and their contribution to the uptake of chaperone-peptide complexes (9.3.1.3). In the following section (9.3.2) we will discuss the role of SR in mediating the immunomodulatory properties of apoptotic cells (9.3.2.1)

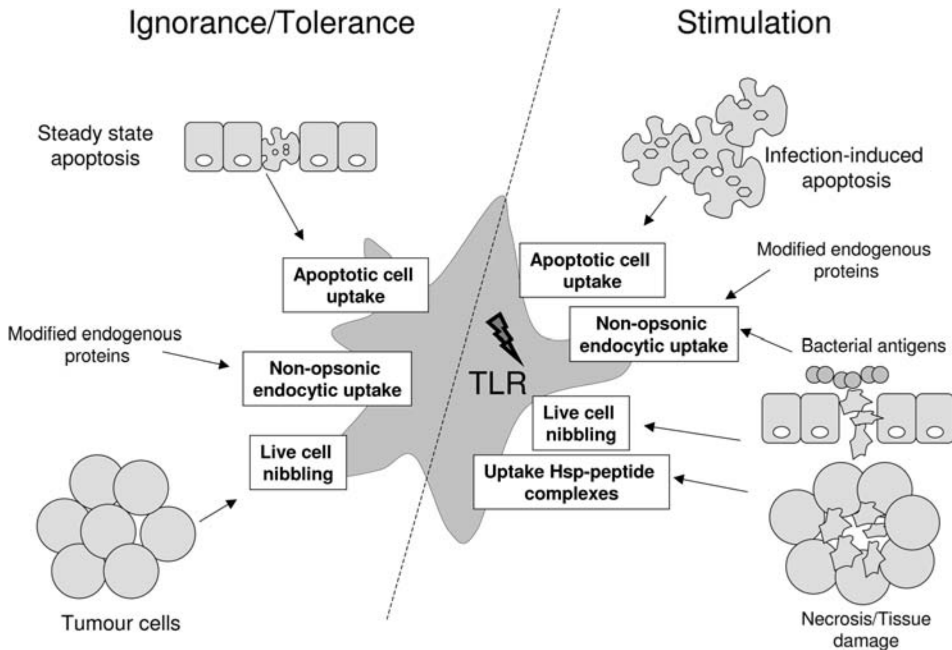

Fig. 9.2 SR function on DC. SR can contribute to several processes that facilitate Ag sampling by DC and that can lead to either immunological ignorance and/or tolerance or to immune stimulation. The outcome would depend on the presence of activators of the innate immune system, such as TLR agonists.

and necrotic cells (9.3.2.2) and in altering TLR-mediated recognition (9.3.2.3). An overview of the processes where SR could be involved is shown in Fig. 9.2.

9.3.1
SR and Antigen Internalization for Presentation to the Acquired Immune System

The broad range of Ag internalization and presentation pathways present in DC provide a useful means to expand the range of molecules presented to the acquired immune system and is not surprising that effective endocytic receptors with a broad binding specificity, such as SR, have also been exploited in this manner by these professional Ag-presenting cells.

Exogenous Ag is acquired by APC by a variety of methods, including receptor-mediated endocytosis, macropinocytosis and phagocytosis. Once inside the cell, Ag are delivered to the endosomal compartment where degradation occurs. Peptides are subsequently loaded into MHC class II molecules and traffick to the plasma membrane. In contrast endogenous Ag are degraded in the cytosol by the proteosome and the resultant peptides are transported into the endoplasmic reticulum where loading onto MHC class I molecules occurs. However exogenous proteins can also gain access to the MHC class I pathway under certain conditions, a process known as crosspresentation [80, 81]. Crosspresentation is important for generating immunity to viruses and for generating tolerance to self Ag. The exact molecular mechanisms that allow this to occur are not fully elucidated at present through it is clear that the presentation of cell-associated Ag is more efficient than of free Ag.

9.3.1.1 *In vitro* Generation of SR Ligands and its Effect in Immunogenicity

SR ligands can be generated through chemical modification of proteins to enhance their negative charge by alterating the ε-amino groups of their lysine residues with acetic or maleic anhydrides, or more physiologically, with malondialdehyde. The effect of these modifications in the imunogenity of protein antigens has been assessed, prompted by the efficient endocytic activity of SR and their expression on APC subsets.

In vivo studies by Abraham et al. showed that maleylation of diphtheria toxoid (DT) induced targeting to SR and enhanced its immunogenicity. Maleylated DT (mDT) generated a significant Ab response. Immunization with soluble mDT elicited a better T-cell proliferative response *in vitro* compared to immunization with soluble DT. This enhanced presentation could be blocked by unrelated maleylated proteins which suggests that enhanced mDT presentation was due to increased receptor-mediated targeting and not APC activation. Even though maleylation did not seem to alter the repertoire of T-cell epitopes, it disrupted the native B-cell epitopes and created new epitopes that were shared between different maleylated proteins. Further work has demonstrated that mice could generate Abs against the maleylated form of a ubiquitous self Ag, mouse serum albumin [82] and that T-cell tolerance to this protein had been broken in these mice.

In a related study Singh et al. showed that spleen cells from mice immunized with maleylated Ag made relatively more IFNγ and less IL-4 or IL-10 than cells from mice immunized with native Ag. This was also reflected in the IgG1:IgG2a ratios of Abs generated *in vivo*. Using a T-cell line to detect peptide-MHC complexes expressed on spleen cells in Ag-injected mice, the authors found that higher levels of these complexes were generated *in vivo* from maleylated proteins and that they persisted longer than those generated from the native protein. Together these data suggest that, in certain situations, the levels of cognate ligand available and/or the time course of their availability may play a major role in determining the cytokine profiles of the responding T cells in addition to costimulatory signals [83].

Bansal et al. showed that maleylation of OVA enhanced its presentation to an OVA-specific MHC class I-restricted T-cell line by both Mϕ and B cells and that this enhanced presentation involved uptake through receptors of SR-like ligand specificity, was TAP-1-independent, and was inhibited by low levels (2 mM) of ammonium chloride [84]. Following a similar approach Shakushiro et al. recently showed that efficient Ag delivery *via* SR resulted in enhanced crosspresentation to a T-cell hybridoma by the murine DC line DC2.4. Targeting to SR was achieved by chemically acylating OVA. Three different acylated proteins were used in this study succinylated OVA (succ-OVA), maleylated OVA (mal-OVA) and *cis*-aconitylated OVA (aco-OVA). These modifications resulted in augmented receptor mediated endocytosis compared to native OVA, but only mal-OVA and aco-OVA were efficiently crosspresented to CD8OVA1.3 T cells. The authors believed that this could be due to the enhanced stability of succ-OVA under acidic conditions whereas both mal-OVA and aco-OVA are deacylated in the endosome. It is also possible that succinyl moieties inhibit ubiquitin conjugation on lysine residues, a necessity for proteasome degradation. Finally the presence of succinyl on the lysine of the SIINFEKL peptide could interfere with T-cell recognition. Although not shown directly, the authors speculate that the SR mediating the uptake of modified OVA is LOX-1 [85].

9.3.1.2 Crosspresentation of Ag Through Uptake of Apoptotic Cells

Albert et al. demonstrated that human immature DC, but not Mϕ, efficiently presented Ag derived from virally infected apoptotic cells, and stimulated class I-restricted $CD8^+$ CTL suggesting a major role for apoptotic cell uptake in the elicitation of CD8-mediated responses [86]. In mouse, $CD8\alpha^+$ DC have been shown to be responsible for crosspresentation [87]. Further studies demonstrated that crosspresentation *per se* is insufficient to induce CD8 T-cells priming because crosspresentation of cellular material by $CD8\alpha^+$ DC has also been implicated in $CD8^+$ T-cell tolerance [88, 89]. These opposing outcomes seemed to be determined by the presence of signals in virally infected cells, absent in their uninfected counterparts, favoring crosspriming. This idea is supported by the requirement of TLR3-mediated recognition of ssRNA on virally infected cells for efficient crosspresentation [90].

A clearly defined change on the apoptotic cell surface is the exposure of phosphatidylserine, a lipid restricted to the inner surface of the plasma membrane in healthy cells. The phosphatidylserine receptor in the membrane of APC, has been

shown to play a major role in apoptototic cell recognition through a direct interaction with phosphatidylserine on the apoptotic cell [91]. Other phosphatidylserine-binding membrane proteins include various SR. A tether and tackle mechanism has been suggested for the uptake of apoptotic cells. According to this model ligation of some apoptotic cell recognition receptors such as SR would induce tethering of cells to APC without uptake and inclusion of phosphatidylserine on the target cell membrane would initiate uptake [52]. As discussed earlier, SR-A and CD36 have been shown to recognize apoptotic cells [16, 47, 48] but in both cases the ligands involved on the apoptotic cells have not been clearly defined. The v integrins: v3 on Mϕ [47] and v5 on DC [48] were closely associated with CD36 activity. In some of these CD36-mediated systems bridging by thrombospondin has been suggested. Navazo et al. identified the domain on CD36 implicated in the phagocytosis of apoptotic neutrophils and located it to residues 155–183 [92].

CD36 expression is higher in $CD8\alpha^+$ cells compared to $CD8\alpha^-$ cells but DC obtained from CD36 deficient mice can still crosspresent Ag [55, 93] indicating that CD36 is not essential for this process. Similarly, regardless of the major contribution of SR-A to clearance of apoptotic thymocytes *in vitro* [16], no defect in apoptotic cell clearance has been observed in thymus of SR-A-deficient mice [17]. These observations suggest that the level of redundancy observed in apoptotic cell recognition *in vitro* [52] could be an indication of the multiple pathways used *in vivo* for apoptotic cell removal and make the assessment of the contribution of individual receptors difficult.

9.3.1.3 Crosspresentation of Ag Acquired through Live Cell Nibbling

DC can crosspresent to $CD8^+$ T cells Ag acquired from live cells *in vitro* through a process called nibbling [94]. The ability to nibble from live cells appears to be exclusive to DC. Immature DC efficiently acquired labeled plasma membrane and intracellular proteins from DC, Mϕ, B cells, activated T cells and tumor cells. This uptake was shown to be receptor specific and could be blocked by the SR ligands fucoidan, polyvinylsulphate and polyG. Anti-class A SR mAb, but not anti-CD36 mAb, partially inhibited the capture and crosspresentation of Ag from live cells suggesting the involvement of other SR [19].

The significance of live cell nibbling *in vivo* has not been determined although it is speculated that it could have an important role in the generation of self-tolerance. However, as no obvious autoimmune disease susceptibility has been reported in the case of SR-A deficient mice it is it likely that there is a great deal of redundancy in the system. As in the case of apoptotic cell uptake, the presence of innate stimulators such as TLR agonists or necrotic cells could favor the presentation of viral and tumor-associated antigens using this mechanism.

9.3.1.4 Crosspresentation of Peptide-chaperone Complexes

Molecular chaperones such as gp96, Hsp70, Hsp90, Hsp110 and calreticulin control the folding of proteins and inhibit their aggregation [95]. Tumor-derived HSP

have been reported to initiate protective CD8-mediated T-cell immunity [96] through binding to tumor-derived peptides [97–99]. The antigenic specificity of HSP was shown to derive not from the HSP molecule *per se*, but from the peptides chaperoned by them. This is a controversial research area as recent evidence has questioned the ability of these molecules, in particular gp96, to bind peptides *in vivo* [100, 101]. Recently Zheng and Li addressed this problem using mice that were defective for heat shock factor 1 (Hsf1), a major transcription factor for HSP. Hsf1 is essential for the induction of inducible HSP, but also for the constitutive expression of Hsp90 and Hsp70. Ag from *Hsf1*$^{-/-}$ cells were unable to gain effective access to the crosspresentation pathway to stimulate T-cell proliferation or to trigger IFNγ and IL-2 release. The differences between WT and *Hsf1*$^{-/-}$ cells were observed *in vitro* and *in vivo*, regardless of the Ag tested. These authors suggest that the complex of HSP with protein Ag rather than the HSP-peptide complex or free peptide/protein Ag is the physiological substrate for the cross-priming pathway and predict that HSP in the cytosol are more important than HSP in other subcellular locations in facilitating crosspresentation, which explains why ER chaperones are dispensable for crosspresentation and why the crosspresentation of *Hsf1*$^{-/-}$ cell-associated Ag is reduced despite normal levels of ER chaperones such as gp96 [102].

Recognition of HSP-peptide complexes by APC is receptor mediated. Once bound, HSP-Ag complexes are internalized into the early and late endosomal compartments where they co-localize with MHC class I molecules [103–105] allowing efficient presentation of the targeted peptides by MHCI. Even though one of the major mechanisms of HSP uptake is recognition through CD91, the α_2-macroglobulin receptor [106], there is evidence suggesting a role for SR in the recognition of selected chaperones. A role for SR-A in chaperone-peptide complex internalization has been proposed by Berwen *et al*. These authors observed that in murine Thioglycollate-elicited Mϕ an OVA peptide internalized as a complex with gp-96 could be presented in the context of MHCI and that complex internalization could be inhibited by fucoidan. Further work demonstrated that SR-A mediated the recognition and internalization of gp96 and CRT in elicited murine Mϕ, a murine Mϕ cell line and human cells expressing SR-A under control of an inducible promoter [18].

Investigation of the SR able to bind Hsp70 demonstrated that Lox-1 expression confers the ability to bind this chaperone and anti-Lox-1 mAb 23C11 substantially inhibited HSP70 binding in human immature DC and Mϕ. Furthermore, anti-Lox-1 mAb inhibited crosspresentation of OVA-Hsp70 *in vitro*. The authors demonstrated that Lox-1-mediated targeting is an efficient system for MHCI targeting *in vivo* by showing that complexes of OVA-anti-Lox1 mAb could induce an antitumoral immune response against an OVA-expressing tumor [61].

The type F scavenger receptor SREC-I has been shown to mediate uptake of calreticulin and gp96 in CHO and RAW264.7 cells. In CHO cells SREC-I-mediated uptake of calreticulin was inhibited by fucoidan and acetylated LDL. These results suggest that SREC-I could mediate the residual internalization of calreticulin observed in SRA–/– Mϕ, which could be inhibited by acetylated LDL [69]. Further

support for a role of SR in chaperone internalization has been provided by the involvement of CD36 recognition of gp96 [107].

9.3.2 Role of SR in the Modulation of Dendritic Cell Activation

9.3.2.1 Role of SR in Modulation of Dendritic Cell Phenotype in Response to Uptake of Apoptotic Cells

Apoptotic cell clearance is a noninflammatory process. Indeed inflammation is actively suppressed, both *in vitro* and *in vivo,* through the production of inhibitory factors such as transforming growth factor (TGF)β, prostaglandin E2 (PGE2) and IL-10 [52]. In agreement with these observations phosphatidylserine containing liposomes had an inhibitory effect on Ag-specific immune responses *in vivo* that were mimicked by an agonistic Ab-specific for the phosphatidylserine receptor and could be reversed by *in vivo* administration of anti-TGFβ Ab [108].

To date the only information available in support of a role for SR in mediating the anti-inflammatory effects from apoptotic cell uptake has been obtained in human DC [56]. In this culture system anti-CD36 and anti α_v (CD51) mAb induced an altered maturation pattern on DC. DC exposed to medium alone, anti-CD36 mAb or apoptotic cells and then matured with LPS secreted TNFα. But anti-CD36 mAb or apoptotic cell treatment abolished IL-12 p70 secretion but induced IL-10 synthesis. Blocking anti-IL-10 mAbs failed to prevent the modulation of DC by anti-CD36 mAb. Treated DC were also unable to activate T cells. Modulation of DC activity by apoptotic cells resembles the effect obtained upon adhesion of *P. falciparum*-infected erythrocytes [109]. This interaction might confer a selective advantage upon the parasite as delay in the elicitation of immune responses could occur through modulation of the function of APC.

9.3.2.2 Role of SR in Modulation of Dendritic Cell Phenotype in Response to Uptake of Necrotic Cells

In contrast to apoptosis, cell death by necrosis is typically associated with inflammation. Immature DC efficiently phagocytose necrotic tumor cells and exposure to necrotic cells induces maturation. Matured DC express high levels of the DC-restricted markers CD83 and lysosome-associated membrane glycoprotein (DC-LAMP) and the co-stimulatory molecules CD40 and CD86. Furthermore, they develop into powerful stimulators of both $CD4^+$ and $CD8^+$ T cells. In the light of the potent stimulatory capacity of chaperones it could be speculated that the potency of necrotic cells could be due to the activity of chaperones released into the extracellular milieu. Therefore the Ag presented by non-fully matured/activated DC upon internalization of apoptotic cells is likely to lead to immunotolerance and that this could be altered in the presence of necrotic cells. In agreement with this, optimal crosspresentation of antigens from tumor cells requires two steps: phagocytosis of apoptotic cells by immature DC, which provides antigenic peptides for major histocompatibility complex class I and class II presentation, and a maturation signal

that is delivered by exposure to necrotic tumor cells, their supernatants, or standard maturation stimuli, e.g. monocyte-conditioned medium. Thus, DC are able to distinguish two types of tumor cell death, with necrosis providing a control that is critical for the initiation of immunity.

It is tempting to consider HSP as "danger signals" released by host cells during tissue damage. Indeed exposure to Hsp70, Hsp60 or gp96 causes secretion of inflammatory cytokines by Mϕ and DC, chemokines by Mϕ and NO by Mϕ and expression of maturation markers and migration to draining lymph nodes by DC [110]. Multiple molecules have been involved in HSP-mediated APC activation such as members of the TLR family, CD40 and CD14. However this is a controversial issue as there are concerns as to whether contaminants such as LPS were mediating the observed effects [111]. Indeed this concern should be also raised in the case of ligand binding studies were LPS-containing preparations of HSP have been used to test binding to SR able to interact with endotoxin.

9.3.2.3 Cross-talk between SR and Toll-like Receptors

SR could be involved in modulating innate responses by facilitating the access of TLR agonists to their receptors. A role for CD36 in innate recognition is supported by the analysis of mice with a nonsense mutation of CD36 induced by *N*-ethyl-*N*-nitrosourea (oblivious). This mutation caused a recessive immunodeficiency phenotype in which Mϕ were insensitive to the *R*-enantiomer of MALP-2 (a diacylated bacterial lipopeptide) and to LTA. Homozygous mice were more susceptible to *Staphylococcus aureus* infection. CD36(obl) macrophages readily detect S-MALP-2, PAM(2)CSK(4), PAM(3)CSK(4) and zymosan, revealing that recognition of some, but not all, TLR2 ligands are dependent on CD36 [53].

In the case of SR-A, no difference has been observed in the TNFα production in response to *N. meningitidis* in bone marrow Mϕ in the absence of SR-A regardless of the lack of bacteria internalization observed in SR-A deficient Mϕ [9]. Recently, using the anti-SR-A mAb 2F8, it has been shown that SR-A ligation in murine Mϕ stimulated H_2O_2 production and inhibited the production of IL-12 in response LPS and IFNγ treatment [112].

9.4 Concluding Remarks

SR comprise a heterogeneous group of receptors that in addition to their role in clearance of modified lipoproteins and atherosclerosis, could influence the range of Ag presented by APC to the acquired immune system and modulate the type of acquired response elicited. This is a vast research area where individual receptor contribution might be masked by functional redundancy but where the cooperation between biochemical, cellular biology and immunological approaches will enrich our understanding of DC heterogeneity and APC potential.

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10
Production of the Long Pentraxin PTX3 by Myeloid Dendritic Cells: Linking Cellular and Humoral Innate Immunity

Paola Allavena, Barbara Bottazzi, Andrea Doni, Luigina Romani, Cecilia Garlanda and Alberto Mantovani

10.1
Introduction

Dendritic cell (DC) function is an important bridge between innate and adaptive immunity [1–3]. Innate defense mechanisms involve cellular and soluble pattern recognition receptors (PRR) [4]. Cellular PRR belong to different functional and structural groups, which include the Toll-like receptors (TLR), scavenger receptors, lectin receptors and G protein coupled receptors for formyl peptides [5, 6]. The soluble arm is also diverse, including collectins (mannose binding lectin, surfactant protein A and D, C1q), ficolins and pentraxins [7, 8].

DC express a number of TLRs and C-type lectin receptors with which they recognize pathogens and damaged tissues. Once activated through these receptors DC release a variety of soluble products which have a crucial role in the orientation and amplification of the immune response. While much has been reported on cellular PRR expressed by DC, little is known about the soluble mediators of innate immunity and their cross-talk with DC. In this chapter we will describe the regulated production of the long pentraxin PTX3 by DC and its role in pathogen recognition and orientation of the immune response, highlighting the complexity and complementarity of cellular and humoral innate immune recognition.

10.2
The Pentraxin Superfamily and the Prototypic Long PTX3: Molecules and Ligands

Pentraxins are a superfamily of evolutionary conserved proteins characterized by a structural motif, the pentraxin domain. C reactive protein (CRP), the first PRR to be identified, and the serum amyloid P (SAP) component, belong to the short pentraxin arm of the superfamily [7–9].

During the early 1990s, a new pentraxin domain-containing secreted protein was identified as an IL-1 inducible gene in endothelial cells (PTX3) [10] or as a TNF-

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stimulated gene (TSG-14) in fibroblasts [11]. The main structural property of the long pentraxin PTX3 which differentiated it from CRP and SAP, was the presence of an amino-terminal domain of 174 amino acids, that was not present in CRP and SAP, coupled to the pentraxin domain (Fig. 10.1).

Available information indicates that PTX3 is an essential component of the humoral arm of innate immunity, activated following pathogen recognition by cellular PRR. PTX3 acts as a functional ancestor of antibodies, recognizing microbes, activating complement, facilitating pathogen recognition by phagocytes, hence playing a nonredundant role in resistance against selected pathogens [12]. Unlike the classic short pentraxins, CRP and SAP, which are produced in the liver, PTX3 is expressed in a variety of tissues and represents a mechanism of amplification of innate resistance against pathogens, acting locally at sites of infection and inflammation.

In addition, PTX3 is essential in female fertility because it acts as a nodal point for the assembly of the cumulus oophorus hyaluronan-rich extracellular matrix [13]. Thus, the prototypic long pentraxin PTX3 is a multifunctional soluble pattern recognition receptor at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility.

As listed in Table 10.1, PTX3 and the short pentraxins CRP and SAP have partially different ligand specificity, thus amplifying the repertoire of the innate immune system. The first described and best characterized ligand of PTX3 is the complement component C1q [14, 15]. Interaction of PTX3 with plastic-immobilized C1q induces activation of the classical complement pathway as demonstrated by an increased deposition of C3 and C4.

Similarly to CRP and SAP, PTX3 binds to apoptotic cells inhibiting their recognition by dendritic cells [16]. Binding occurs late in the apoptotic process and, in contrast to what happens for classic short pentraxins, is calcium-independent. In addition preincubation of apoptotic cells with PTX3 enhances C1q binding and C3

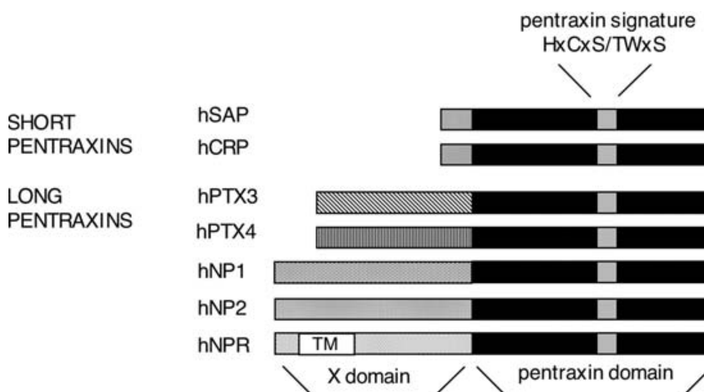

Fig. 10.1 The pentraxin superfamily in humans. Short pentraxins: C reactive protein (CRP); serum amyloid A (SAP). Long pentraxins, among which PTX3. TM: transmembrane domain.

Tab. 10.1 Differential ligand recognition by PTX3 and short pentraxins.

	PTX3	*CRP*	*SAP*
Membrane moieties:			
PC	–	+	–
PE	–	–	+
Galactomannan*	+	NT	NT
LPS	–	–	+
OmpA	+	NT	NT
Complement components:			
C1q	+	+	+
C4b-binding protein	–	–	+
Matrix proteins:			
TSG6	+	NT	NT
Laminin	–	+	+
Type IV collagen	–	–	+
Fibronectin	–	+	+
Chondroitin sulfate	–	NT	+
Hyaluronic acid	–	NT	NT
Microbes:			
Aspergillus fumigatus	+	+	NT
Pseudomonas aeruginosa	+	NT	NT
Salmonella typhimurium	+	–	+
Paracoccdioides brasiliensis	+	NT	NT
Zymosan	+	+	+
Miscellaneous:			
Apoptotic cells	+	+	+

* based on competition data.

deposition on the cell surface, suggesting a role for PTX3 in the complement-mediated clearence of apoptotic cells [15].

Selected pathogens are bound by PTX3, including conidia of *Aspergillus fumigatus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Paracoccidioides brasiliensis* and zymosan. No significant binding was found with *Escherichia coli*, *Burkholderia cepacia*, *Listeria monocytogenes* or *Candida albicans* [17, 18].

The microbial moieties recognized by PTX3 have not been completely defined (Table 10.1). PTX3 does not bind LPS nor the classical short pentraxin ligands (PC, phosphoethanolamine (PE) and high pyruvate agarose). Binding of conidia of *A. fumigatus* is in competition with galactomannan. Mannan recognition is consistent with binding to zymosan. We recently identified outer membrane protein A (OmpA), as a bacterial moiety specifically bound by PTX3 [19].

Matrix components (fibronectin, type IV collagen) are recognized by SAP, but not by PTX3. In contrast PTX3 binds the matrix component TNFα-induced protein 6 (TNFAIP6 or TSG6) [13] a multifunctional protein usually associated with in-

flammation [20]. By binding to TSG6, PTX3 acts as a nodal point for the assembly of hyaluronic acid rich extracellular matrix, essential for female fertility [20].

PTX3 binds fibroblast growth factor 2 (FGF2), but not other members of the FGF family or cytokines and chemokines. The angiogenic activity of FGF2 *in vitro* and *in vivo* is blocked by PTX3 [21].

10.3
Myeloid DC as a Major Source of PTX3: Regulation of Production

PTX3 production is induced by primary inflammatory signals, such as IL-1, TNF and microbial moieties (e.g. LPS, lipoarabinomannans) [10, 12]. Specifically, agonists for different members of the TLR family stimulate PTX3 production [22] (and see below). Unlike CRP and SAP, no induction of PTX3 is observed with IL-6 [10, 12].

A variety of cell types can produce PTX3 *in vitro* upon exposure to primary inflammatory signals. These include endothelial cells, smooth muscle cells, adipocytes, fibroblasts, mononuclear phagocytes and dendritic cells (DC) [10, 12]. We have recently reassessed the relative capacity of different cell types to produce PTX3, and found that dendritic cells produce the highest amounts [22].

Because DC have a central role in innate and adaptive immunity, we characterized the *in vitro* and *in vivo* production of PTX3 by human and mouse DC, and defined the role of different innate immunity receptors in its induction.

DC were by far the best producers of PTX3 after LPS stimulation, when compared with undifferentiated monocytes and *in vitro*-derived macrophages [18]. In selected experiments of *in vitro* infection with *A. fumigatus*, PTX3 production was again higher in DC compared to T lymphocytes and primary cultures of fibroblasts, endothelial and epithelial cells [18].

Beside LPS, which engages TLR4, other TLR ligands triggered the production of PTX3. TLR activate a similar signaling pathway [5], but the diverse members of the TLR family differ in their capacity to activate collateral signaling molecules (e.g. MAL/TIRAP) and to induce production of downstream effectors [23]. Myeloid DC express a wide spectrum of TLR [24, 25]. It was therefore important to assess whether different TLR agonists induce production of PTX3 in DC.

PTX3 was significantly induced by activation Omp A (TLR2), PGN (TLR2), poly (I):(C) (TLR3), *Candida* (TLR4) and flagellin (TLR5). As expected, CpG ODN 2006 did not induce PTX3 production in DC (Table 10.2), consistently with the low expression of TLR9 in myeloid DC. As PTX3 is a NF-kB-regulated gene [26] the finding that agonists of different TLRs all induced protein production suggests that PTX3 is downstream of the common core signaling MyD88 pathway.

Production of PTX3 by myeloid DC precursors (mononuclear phagocytes) after exposure in vitro to Th1 and Th2 cytokines, has also been studied [12]. IFNγ inhibits PTX3 expression and production in different cellular contexts [27]. IL-4 and IL-13 do not affect PTX3 production whereas, surprisingly, IL-10 was found by transcriptional profiling to induce PTX3 expression in dendritic cells and monocytes

Tab. 10.2 PTX3 production by *in vitro* differentiated and blood circulating myeloid and plasmacytoid DC upon triggering with different TLR ligands.

	Stimuli						
Exp.1	**None**	**PGN**	**poli I:C**	**LPS**	**OMP A**	**Candida**	**Flagellin**
In vitro DC	<0.05	14	28	45	20	6	2.5
Exp.2	**None**	**PGN**	**poli I:C**	**LPS**	**A. fumig.**	**P. aerugin**	
In vitro DC	<0.05	10	37	50	29	53	
Blood MyDC	<0.05	5	3	6	1,5	2	
Blood pDC	<0.05	NT	0.5	0.3	<0.05	0.2	
Blood pDC	**CpG ODN 2006**	**CpG ODN**	**Influenza virus**				
	<0.05	<0.05	<0.05				

In vitro DC were differentiated from blood monocytes cultured with GM-CSF and IL-13 (22). MyDC : myeloid DC isolated from blood with magnetic beads coated with mAb BDCA-1 pDC : plasmacytoid DC isolated from blood with magnetic beads coated with mAb CD123 and BDCA-4.
Results are values of PTX3 (ng ml^{-1}) measured by Elisa.

and to costimulate PTX3 production with LPS ([28] and Doni unpublished data). PTX3 is therefore part of the genetic program expressed by M2 mononuclear phagocytes and IL-10-treated DC. As mentioned above, PTX3 has a role in matrix organization; therefore its expression by IL-10 is likely to be related to the orchestration of matrix deposition, tissue repair and remodelling, processes that are regulated by IL-10 [28, 29].

10.4 Blood-circulating Myeloid, but not Plasmacytoid, DC Produce PTX3

DC are heterogeneous and pDC represent a well defined cell population present in blood and lymphoid tissues with distinct surface phenotype and functional properties [30, 31]. It was therefore important to ascertain whether circulating MyDC (HLA-DR^+, Lin^-, $CD11c^+$, $CD19^-$, $CD1a^+$ and BDCA-1^+) have the capacity to produce PTX3 and whether this property is shared by pDC ($CD4^+$, $CD11c^-$, Lin^-, $CD123^+$ and BDCA-4^+). As shown in Table 10.2, exposure to LPS, PGN, poly (I):(C) or whole *A. fumigatus* conidia or bacteria (*P. aeruginosa*) induced PTX3 production in freshly isolated MyDC [22], consistently with their ample repertoire of functional TLR [24]. pDC did not produce PTX3 in response to the same signals [22], as expected on the basis of defective expression of TLR2, TLR3, TLR4, TLR5 and TLR8 [25, 32]. Unlike MyDC, pDC have high levels of TLR7 and TLR9 in humans. However, CpG ODN 2006 (TLR9) did not induce PTX3 production in pDC. Similarly, exposure to influenza virus failed to cause PTX3 production in pDC. It should be emphasized that under the same conditions CpG ODN and influenza virus induced copious amounts of selected chemokines (not shown).

Overall, DC of myelomonocytic origin are the most efficient producers of PTX3, on a per-cell basis, among immunocompetent cells [22]. Interestingly, production of PTX3 is restricted to myeloid DC, while interferon-producing plasmacytoid DC are unable to produce PTX3 in response to appropriate agonists. PTX3 production by DC and neighboring macrophages is likely to facilitate pathogen recognition and activation of an appropriate adaptive immune response.

10.5 Role of PTX3 in Innate Immunity

The investigation of *ptx3*$^{-/-}$ mice, generated by homologous recombination, has been invaluable in understanding the *in vivo* function of PTX3 [13, 18]. *Ptx3*-deficient mice are viable and display a normal life span in a conventional mouse facility. The only apparent abnormality is a severe deficiency in female fertility, as discussed elsewhere [13].

Among microbes recognized by PTX3 (Table 10.1), we focused attention on *A. fumigatus* because this fungus is a major pathogen in immunodeficient individuals.

In vivo experiments clearly demonstrated that *Ptx3*$^{-/-}$ mice were extremely susceptible upon intratracheal infection with *A. fumigatus* in terms of mortality, number of lung colonies and lung inflammatory response [18]. The specificity of the role played by PTX3 in the susceptibility to *A. fumigatus* infection could be demonstrated by the complete protective effect of *ptx3*$^{-/-}$ mice by exogenous PTX3 treatment [18].

It should be noted that susceptibility to other pathogens, not recognized by PTX3, including for instance *L. monocytogenes,* was not increased in *ptx3*$^{-/-}$ mice. Moreover, in models of intra-abdominal sepsis caused by caecal ligation and puncture, *ptx3*$^{+/+}$ and *ptx3*$^{-/-}$ mice behaved similarly ([18] and unpublished results). These results suggested that PTX3 deficiency does not cause a generalized impairment of host resistance to microbial pathogens, and that PTX3 is involved in recognition of specific microorganisms.

10.6 Function of Antigen Presenting Cells in PTX3-deficient Mice

It is well established that resistance to *A. fumigatus* requires the endocytic activity of phagocytes and is associated with the activation of a polarized type 1 T-cell response [18, 33, 34].

In vitro, the ability of alveolar macrophages to ingest and kill resting conidia was impaired in *Ptx3*$^{-/-}$ mice, as compared to intact mice. PTX3 restored both the phagocytic and conidiocidal activities of cells from *Ptx3*$^{-/-}$ mice [18].

Also in murine mononuclear phagocytes and DC, *A. fumigatus* conidia rapidly induced in vitro PTX3 production. These *in vitro* results found support from *in vi-*

vo evidence: PTX3 plasma levels were increased in mice infected with *A. fumigatus* and in neutropenic patients with systemic infection with *A. fumigatus* [18]. Overall these experiments indicate that PTX3 production, induced in immunocompetent cells upon selected pathogen recognition, facilitate pathogen uptake and clearance.

To better understand which mechanisms were defective in *Ptx3*$^{-/-}$ mice, cytokine levels were measured in the lungs of mice infected with *A. fumigatus* conidia. IFN-γ levels were reduced and IL-4 levels were increased in *Ptx3*$^{-/-}$ mice compared to control animals. This defect was reverted by the administration of recombinant PTX3. Moreover, while lung DC from intact mice produced IL-12 upon recognition of *A. fumigatus* conidia, DC from *Ptx3*$^{-/-}$ mice did not. Addition of PTX3 fully restored the ability of *Ptx3*$^{-/-}$ DC to respond to conidia by producing IL-12 [18].

Collectively, these results suggest that, by favoring recognition of conidia, PTX3 facilitates macrophage-mediated resistance against pathogens as well as activation by DC of a protective type 1 antimicrobial response, as depicted in Fig. 10.2.

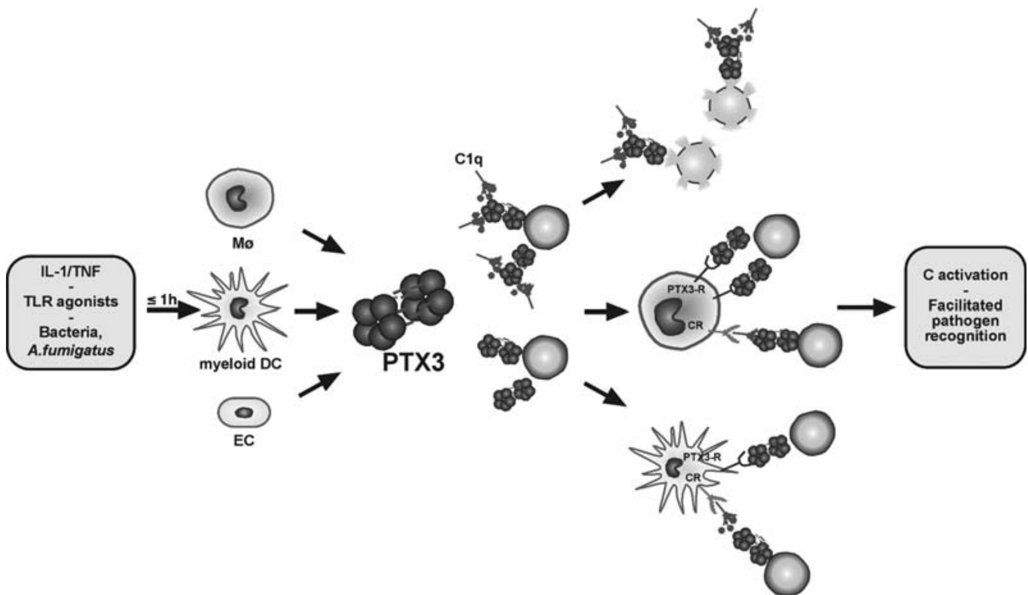

Fig. 10.2 The role of PTX3 in innate immunity. PTX3 produced by myeloid DC and other cell types is induced by inflammatory cytokines, TLR agonists and selected pathogens. PTX3 activates complement and facilitates pathogen recognition, thus linking cellular and humoral innate immunity.

10.7
Conclusion

Analysis of gene-targeted mice has revealed that PTX3 is a unique fluid phase pattern recognition receptor, which plays an important role in antimicrobial resis-

tance [18]. Unlike short pentraxins which are mainly produced in the liver, PTX3 is secreted by several cell types, and in particular by myeloid DC upon triggering via members of the TLR family. PTX3 binds selected pathogens (e.g. conidia of *A. fumigatus*; *P. aeruginosa*) and activates at least two effector pathways: by binding C1q, it activates the classic pathway of complement activation; by interacting with unidentified cellular receptor(s) it facilitates pathogen recognition and disposal by macrophages. Moreover, by bridging pathogens and antigen presenting cells, PTX3 influences the activation and orientation of adaptive immunity. Thus, by producing PTX3 in response to TLR engagement, DC activate key component of humoral innate immunity, which acts as a non redundant amplification loop of innate resistance.

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11
Gene Profiling of Dendritic cells during Host–Pathogen Interactions

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11.1
Dendritic Cells as Sentinels of the Immune System: Tissue-resident DC and Migratory DC

Dendritic cells (DC) are bone marrow-derived cells that populate all lymphoid organs including thymus, spleen, and lymph nodes, as well as nearly all nonlymphoid tissues and organs. DC are members of the innate immune system in that they can respond to danger in the host environment by immediately generating protective cytokines.

Human and murine DC have been classified into two main lineages: myeloid DC and lymphoid DC. The definition of dendritic cell subset phenotypes and the attribution of specific functions to defined DC stages has been a very difficult task. DC are characterized by a very high functional plasticity and can adapt their responses upon antigen encounter; in time they are able to segregate different functions, which will dictate the outcome of the immune response.

First, when DC are located in nonlymphoid tissues, close to the mucosal surfaces, they sample the environment to sense the infectious agents. For this task DC use a broad innate receptor repertoire and their phagocytic activity. These cells are named myeloid immature DC and as they are mostly resident in those tissues where they have originally seeded, we will refer to them as tissue-resident DCs (R-DC). Cells that have been conditioned by the microbial encounter migrate to the lymph nodes (LN) where antigen is presented to specific T cells and where initiation of acquired immunity takes place. Thus, mature migratory DC (M-DC) derive from the immature R-DC, and are characterized by a limited degree of plasticity, a limited life span and by the activation of an irreversible differentiation program ending with apoptotic cell death (Fig. 11.1).

The functional properties of immature R-DC are mainly characterized by the ability to sense the environment and to sample microbes at the mucosal sites. In-

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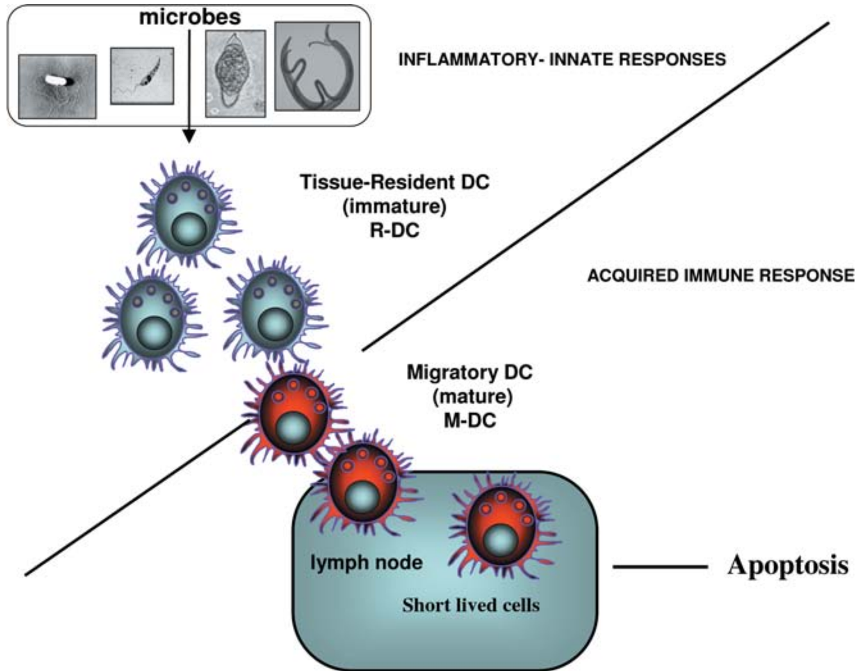


Fig. 11.1 Dendritic cells (DCs) can be divided into two main subpopulations: the resident dendritic cells (R-DC) found in the tissues where they are seeded and the migratory dendritic cells (M-DC) that will migrate to the lymph node upon antigen encounter. Immature R-DC are strategically located in tissues that represent pathogen entry routes, where they continuously monitor the environment through the uptake of both particulate and soluble products. DC maturation is associated with acquisition of migratory functions that allow antigen-loaded DC to move from the marginal zones to the T-cell areas or from nonlymphoid to lymphoid tissues. Mature migratory DC have abundant cell surface major histocompatibility complex (MHC) and co-stimulatory protein expression, have the ability to activate both CD8 and CD4 T cells and undergo cytoskeleton rearrangements that lead to the inhibition of the phagocytic activity and are programmed for apoptotic death.

deed, R-DC are particularly abundant in the respiratory tract and in the lungs, as well as in the gut where, in addition to the Peyer's patches, they can be found in the lamina propria of the intestinal villi. We have shown that in order to sample the gut lumen and sense the intestinal flora, R-DC are able to extend cytoplasmic protrusions in the lumen by opening the tight junctions of the epithelial cells and to preserve the integrity of the epithelial barrier by expressing, in a regulated way, tight junction proteins, such as the occludin [1]. In the skin the R-DC, named Langerhans cells, form a tight network of cells continuously monitoring this tissue for invading parasites [2]. Finally, in the liver and in the spleen where blood-derived antigens are continuously brought and sampled, resident DC are also particularly abundant.

Among the innate receptors, DC express the Toll-like receptor (TLR) family members that bind a variety of microbial ligands [3, 4]. The signaling through

these receptors is mediated either via the MyD88 adaptor molecule, or via an independent pathway involving the TRIF molecule and the IRF-3 transcription factor [5]. As a result of microbial activation through TLRs the NFkB family members are activated and translocated to the nucleus [6] inducing the transcription of many NFkB-dependent genes, mostly immune and inflammatory genes such as cytokines and chemokines. In addition, the microbial activation may also lead to the transcription of the interferon-inducible genes via IRF-3 [7].

R-DC can also display surface molecules such as the MARCO receptor that we have shown to be involved in a profound re-modeling of the actin cytoskeleton [8]. Other receptors expressed by R-DC are DC-SIGN and C-type lectin receptors capable of binding a variety of microorganisms, including viruses such as HIV and pathogenic bacteria such as *Mycobacterium tuberculosis* [9, 10]. Finally, R-DC express the receptors for opsonized microbes such as FcR and CR that have a key role in the phagocytosis process.

11.2 Study of the Complexity of the Immune System using Gene Profiling

The immune response is extraordinarily complex, it involves dynamic interaction of a wide array of tissues, cells and molecules. Traditional approaches are based on one by one gene analysis, shying away from complexity, but providing detailed knowledge of particular molecular entities. The completion of draft sequences of the human and mouse genomes offers many opportunities for gene discovery in the field of immunology through the application of the methods of computational genomics. In concert with emerging genomic and proteomic technologies, it permits the definition of the biology of the immune system. The initiation and regulation of the immune response is complicated and occurs on many levels. Multicellular organisms have been obliged to develop multifaceted innate and adaptive immune systems to cope with the challenges to survival originating from microorganisms and their products. The diversity of innate immune mechanisms is in large part conserved in all multicellular organisms [11]. Some basic principles of microbial recognition and response are emerging, and recently, the application of computational genomics has played an important role in extending such observations from model organisms, such as *Drosophila*, to higher vertebrates, including humans.

The analysis of gene expression in tissues, cells, and biological systems has evolved in the last decade from the analysis of a selected set of genes to an efficient high throughput whole-genome screening approach of potentially all genes expressed in a tissue or cell sample. Development of sophisticated methodologies such as microarray technology allows an open-ended survey to identify comprehensively the fraction of genes that are differentially expressed between samples and define the samples' unique biology. This discovery-based research provides the opportunity to characterize either new genes with unknown function or genes not previously known to be involved in a biological process.

Microarrays were developed in 1995 [12] and have now been widely applied in the field of immunology. Two types of microarrays are commonly used: two-color microarrays and oligonucleotide microarrays. In a two-color microarray, collections of DNA samples (i.e. expressed sequence tag [ESTs] or other clones) are deposited onto a glass slide using robotics. These microarrays are highly flexible as they may be constructed from anonymous clones found in genomic, subtractive, differentially displayed or normalized libraries or from commercially synthesized long ($n = 50$–70) oligonucleotides [13]. Oligonucleotide arrays are constructed from 25-mer oligonucleotides synthesized *in situ* on a solid substrate [14]. This type of microarray requires exact sequence information and bioinformatic design prior to the construction of the microarray. To date, oligonucleotide microarrays cannot be produced in-house and must be purchased from commercial sources. They are still expensive enough to limit the number and scale of experiments that can be performed by a typical laboratory.

However, oligonucleotide microarrays are highly consistent and offer sequence-specific detection of gene expression, which is especially important in the study of gene families. With both types of microarray analysis, data aggregation from multiple experiments is possible, allowing higher order analyses of transcript profiles.

Large-scale gene expression analysis is of great relevance in the field of immunology to generate a global view of how the immune systems attacks invading microorganisms, maintains tolerance, or creates a memory for past infections. Besides availability of large-scale or full-genome microarrays, specialized microarrays that contain a tailored set of DNA sequences related to immunology have been generated and used. Fundamental questions in immunology address how the immune system distinguishes between self- and non-self, and how immune cell differentiation and growth is regulated. The exciting part of microarray studies is that the many data points that are generated cause unpredictable and unexpected results, which may lead to new insights in immunology.

The study of host–pathogen interactions is instrumental for the control of infectious diseases. Host eukaryotes are constantly exposed to attacks by microbes seeking to colonize and propagate in host cells. To counteract them, host cells utilize a whole battery of defense systems to combat microbes. However, in turn, successful microbes evolved sophisticated systems to evade host defense. As such, interactions between hosts and pathogens are perceived as evolutionary arms races between genes of the respective organisms [15, 16, 17]. Any interaction between a host and its pathogen involves alterations in cell signaling cascades in both partners, that may be mediated by transcriptional or post-translational changes. The basic challenge is how to select target genes to be studied in detail from among thousands of genes encoded in the genome. Transcriptomics is one of the methodologies which serves this purpose. Analytical techniques for transcriptomics include differential display (DD [18], cDNA-AFLP [19], random EST sequencing [20] microarray [12], serial analysis of gene expression (SAGE) [21] and massively parallel signature sequencing (MPSS) [22]. Among them, microarray has recently been used more frequently than other platforms. Most of the gene expression studies addressing host–pathogen interactions in reality examined either host or patho-

gen separately. However, the simultaneous monitoring of gene expression of both host and pathogen preferably during the infection process and *in situ*, is at stakes and has already been investigated [23]. This approach is necessary to elucidate the host–pathogen interplay in molecular detail.

11.3 Discovery of IL2 Production by DC using Global Technologies and the NK-DC Interplay

Using the microarray technology, we have recently found that mouse DC are able to produce IL-2 upon bacterial encounter [24]. As well as the other inflammatory cytokines produced by DC during the maturation process, IL-2 is also expressed with a strictly defined kinetic, between two and eight hours after bacterial uptake. IL-2 is a cytokine able to sustain T, B and natural killer (NK) cell growth and, during the late phases of antigen-specific T-cell responses, it contributes to the maintenance of T-cell homeostasis by promoting activation induced cell death (AICD) of effector T lymphocytes [25]. Given the important regulatory role exerted by IL-2 in the immune system, IL-2 deficient mice show a generalized immune system deregulation [26]. The observation that DC, other than T cells, can also produce IL-2 opens new possibilities in understanding the mechanisms by which DC control innate and adaptive immunity [27]. *In vivo*, both $CD8\alpha^{+}$ and $CD8\alpha^{-}$ splenic DC can produce IL-2 following microbial activation. Interestingly, only microbial stimuli and not inflammatory cytokines are able to induce IL-2 secretion by DC [28], indicating that DCs can distinguish between the actual presence of an infection and a cytokine-mediated inflammatory process [29].

Recent studies have focused on the function of DC during the early phases of the immune response, and a predominant role for DC in activation of NK cells has been described [30–33].

NK cells are specialized lymphocytes of the innate immune system capable of eliciting responses against pathogen-infected and tumor cells. They are activated during the early phases of an immune response, a few hours after infection. The functions of NK cells are regulated by a balance of activating and inhibiting signals. These signals are transmitted by inhibitory receptors, which bind class I major histocompatibility complex (MHC) molecules, and activating receptors, which bind ligands on tumors and pathogen-infected cells. Other than surface receptors, cytokines, such as IL-2, IL-12, IL-18 and type I interferons (IFNs) have been shown to promote NK cell priming [34].

Recently, a predominant role of DC in NK cell activation has been described in mouse and human [35]. Both immature and mature human monocyte derived DCs and mouse bone marrow derived DCs have been shown to be able to prime NK cell cytolytic activity [30–32]. This process requires cell-to-cell contact and is independent from IL-12 and IL-2 [32]. DC-NK cells' cross-talk is bidirectional since IL-2 activated NK cells induce immature DC activation in terms of upregulation of co-stimulatory and MHC molecules and inflammatory cytokine production. As

well as DC-mediated NK cell activation, NK-mediated DC activation also requires cell-to-cell contact and mediators produced by NK cells and DC, such as TNFα [32]. Immature DC can prime NK cell activity provided that they are cultured in presence of IL-4 but not if propagated only with GM-CSF [33]. Potential sources of IL-4 *in vivo*, during the early innate phase of the immune response, are NKT cells, basophils and mast cells that have been recently shown to play a critical role in innate immunity to bacterial infections [36–38]. Moreover, basophils and mast cells also produce large amounts of TNFα after bacterial and parasite interaction. TNFα is an additional cytokine able to confer on DC the ability to activate NK cells [33].

Beside these new mechanisms described for NK cell priming, a classical method to activate NK cells *in vitro*, and to increase their antitumor effectiveness, involves culturing NK cells in the presence of IL-2. However, this cytokine has never been considered important *in vivo* for NK cell mediated antitumor or antimicrobial responses as it was believed that IL-2 was exclusively produced by T cells during the late, antigen-specific, phase of the immune response, when the peak of NK cell activation was already exhausted [39]. Nevertheless, as it is clear now that DC are able to make IL-2 early after infection, this assumption has been revised. Indeed, we have shown that after bacterial infection the IL-2 produced by DC is required to elicit IFNγ production by NK cells, at least in mice. As human DC are also capable of producing IL-2 in an IL-15-dependent manner [40], it remains to be investigated if IL-2 could also play a role in stimulating NK cells in humans. The biological relevance of NK cell activation mediated by DC during bacterial infections resides mainly in the secretion of IFNγ [41], which represents the principal phagocyte-activating factor [41, 42]. With regards to antitumor activities, the interaction between activated DC and NK cells has been reported to increase the efficiency of NK cell antitumor effector functions both *in vitro* and *in vivo* in two independent experimental models [33, 43].

We propose that two possible pathways of NK cell priming by DC may exist, one dependent on IL-4 and the other one dependent on microbial stimuli and IL-2. Hence, an appropriate cytokine milieu can render DC competent for NK cell activation independently from the presence of microbial stimuli and IL-2 or, alternatively, following microbial encounter, DCs acquire the ability to activate NK cells with an IL-2-dependent process.

11.4
Profiling of Pathogens and Cells of the Innate Response: Mucosal Epithelial Cells, Phagocytic Cells (Neutrophils and Macrophages)

The interactions between a host and microbial pathogens are diverse and regulated. The molecular mechanisms of microbial pathogenesis show common themes that involve families of structurally- and functionally-related proteins such as adherence factors, secretion systems, toxins and regulators of microbial pathogens. The interaction between pathogen and host uncovers unique mechanisms and molecules. Pathogenesis entails not only the differential expression of bacterial

genes, but also responses by the host. Microarray expression analysis of pathogen-infected cells and tissues can identify, simultaneously and in the same sample, host and pathogen genes that are regulated during the infectious process.

A major challenge to innate immune cells is the discrimination of foreign pathogens from self. As originally described by Janeway [44, 45], innate immune cells possess germline-encoded pattern recognition receptors (PRRs) that recognize and are triggered by evolutionary conserved molecules essential to pathogen function but absent in the host. These pathogen-associated molecular patterns (PAMPs) are widespread and include cell wall components such as mannans in the yeast cell wall, lipopolysaccharide (LPS) in gram-negative bacteria, lipoproteins, peptidoglycans and DNA containing unmethylated CpG motifs. There are at least two distinct classes of PRRs: those that mediate acute phagocytosis as scavenger receptors and mannose receptors, and those that cause immediate cell activation such as TLRs. Upon cellular pathogen uptake, members of the TLR family become recruited to early phagosomes to screen their content for ligands from foreign pathogens and subsequently to trigger cell activation upon ligand recognition [46]. These two activities (internalization versus cell activation) between scavenger receptors and TLRs bear a caveat, given that crosslinking of the scavenger receptor CD36 profoundly modulates LPS-driven DC maturation [47].

Recent studies have shown a stereotyped range of host immune responses after infection with phylogenetically diverse organisms.

Both bacterial and mammalian (mouse, human) genome sequences can be used in microarray technology to define the expression profile of pathogens and the host cells. The global transcription effects on host cells of the innate immunity by various bacterial pathogens, including *Listeria monocytogenes*, *Salmonella*, *Pseudomonas aeruginosa*, and *Bordetella pertussis* have been analyzed by using microarray technology [48]. The infection of macrophages with *S. typhimurium* identified novel genes whose level of expression are altered [49]. Similarly, *L. monocytogenes*–infected human promyelocytic THP1 cells identified 74 upregulated RNAs and 23 downregulated host RNAs [50].

Many of the upregulated genes encode pro-inflammatory cytokines (e.g. IL-8, IL-6, and growth-related oncogene-1), and many of the downregulated genes encode transcription factors and cellular adhesion molecules. Understanding the molecular basis of the host response to bacterial infections is critical for preventing disease and tissue damage resulting from the host response. Furthermore, an understanding of host transcriptional changes induced by the microbes can be used to identify specific protein targets for drug development.

PBMCs transcriptome have been studied by using cDNA microarrays after *in vitro* stimulation with killed *B. pertussis*, *Staphylococcus aureus*, and *Escherichia coli* [51]. This study shows a core of 205 commonly expressed genes. These genes included those with both systemic and local effects. Highly represented were genes encoding intercellular immunoregulatory and signaling molecules such as cytokines and chemokines. These genes are regulated by NFkB, which orchestrates both innate and adaptive immune responses. Gram-negative bacteria induced stronger expression than gram-positive bacteria. Moreover, the study describe 96

genes that were commonly repressed after a delay of about 2 h: a subset of monocyte-attracting chemokines, genes involved in cell–cell adhesion, diapedesis, and leucocyte extravasation, and those involved in recognizing bacteria and antigen presentation.

Therefore, it is possible to distinguish between different species and even individual strains of *B. pertussis* and *S. aureus*. The same group also showed that different expression responses to the same strain of *B. pertussis* depended on whether it was live or killed and, if live, whether it carried a toxin gene [52]. Also the host response to extracellular and intracellular parasites can be assessed by microarrays [53–55]. The gene expression program in response to *Trypanosoma cruzi* infection showed that while 106 genes were expressed at 24 h, none were induced more than twofold by 2 or 6 h.

Microarray technology can provide insights into the interaction between the pathogen and host by revealing global host expression responses to a range of pathogenic stimuli. Pathogens may manipulate host–cell gene expression, for example by causing upregulation of cellular support for pathogen replication and downregulation of MHC expression to allow pathogens to evade the immune system [56, 57].

In conclusion, the amount of data generated by microarray experiments is enormous. A simple experiment comparing stimulation of immune cells by two different bacteria in two individuals at three different time points requires at least 12 microarrays.

With up to 20 000 genes on an array, the number of data points leaps to 240 000. Such quantities of data require specialized statistical expertise and software to decipher patterns from the entire expression repertoire. The bottleneck in genetic analysis has therefore moved from the speed at which an experiment can be done to the speed at which the resulting data can be analyzed.

11.5 Dendritic Cells and Pathogen Interaction: Dendritic Cells as a Link between Innate and Acquired Immunity

When higher organisms are exposed to pathogenic microorganisms, innate immune responses occur immediately, both in terms of cell activation and inflammation. The initial response is characterized by phagocytosis or endocytosis and subsequent destruction or degradation of pathogens.

At the initial stage of primary infection, DC constitute an integral part of the innate immune response, supported by the recruitment activity of bone-marrow-derived immune cells and various resident tissue cells.

DC and macrophages are acutely activated, during innate responses, they produce pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNFα) and interleukin (IL)-1β], and effector cytokines, such as IL-12 (p40 subunit) and type I interferons [58]; this cytokines and chemokines production occurs in waves at precise time point during the process of DC maturation as shown by

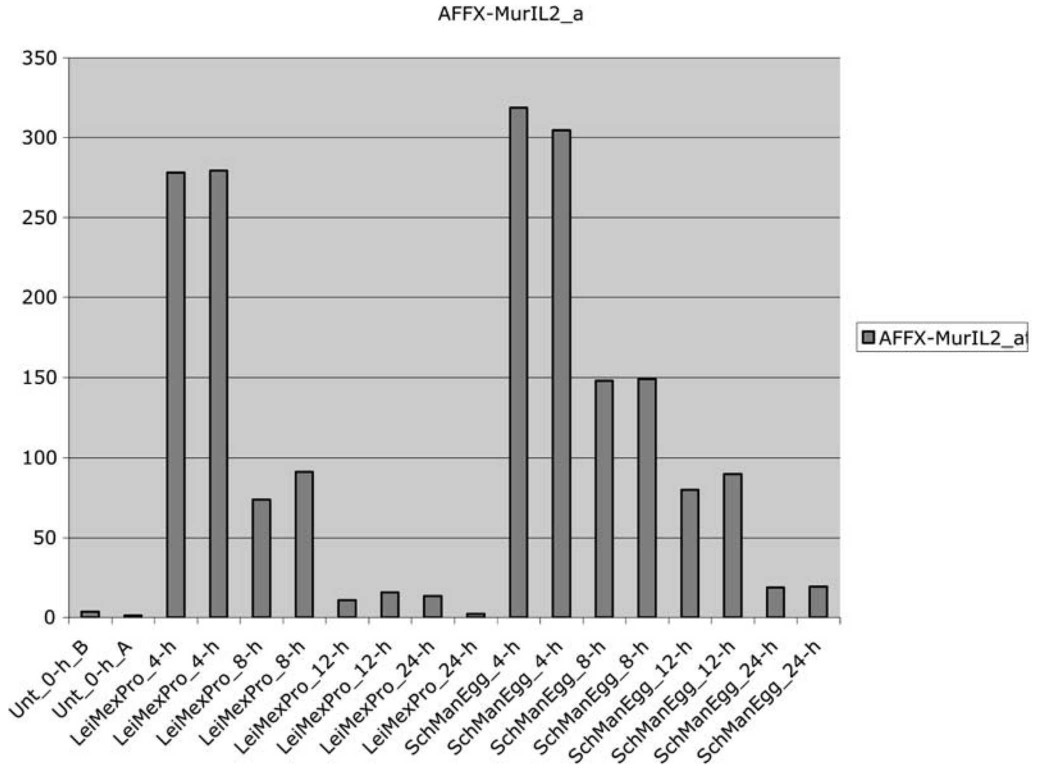


Fig. 11.2 IL-2 gene expression profiling as measured by microarray analysis. The highest level of Il-2 mRNA production is between 4 and 8 h of parasite stimulation. Unt, untreated cell; LeiMexPro, *Leishmania mexicana* promastigote; SchManEgg, *Schistosoma Mansoni* eggs.

the IL-2 gene expression profiling Fig. 11.2. During this phase DC enhance presentation of the products of pathogen degradation (antigenic peptides) via the MHC class I or II presentation pathway to antigen reactive T cells and they produce bactericidal effector substances such as nitric oxide. Thus, innate immune cells and in particular DCs not only represent a first line of defense towards infections but also play an instructive role in shaping the adaptive immune responses [59].

Another interesting feature of R-DC is the ability to delay the processing of the internalized antigens by antigen retention in a storage compartment that we have identified for its mildly acidic pH content [60]. In these vesicles the internalized antigens are not immediately degraded and the fusion with the lysosomes is delayed; this mechanism is apparently coordinated with the generation of newly synthesized MHC class I molecules that occurs 12–18 h following DC activation [61]. How degraded antigens derived from the exogenous pathway can access the MHC class I loading compartment has recently been reported with the discovery of ER-phagosome fusions [62]. Therefore, R-DC can efficiently present on class I molecules peptides generated by the exogenous pathway.

Adaptive immunity is controlled by the generation of MHC-restricted effector T cells and production of cytokines [63]. DC are able to stimulate naïve T helper (Th) cells which in turn may differentiate into Th1- versus Th2-polarized subsets; Th1 cells primarily secrete interferon IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Upon activation, DC upregulate the expression of co-stimulatory molecules, such as CD80 and CD86, thereby increasing immunogenicity of peptide antigens presented. Finally, DC activation triggers production of cytokines, such as IL-12, IL-18, IL-4 or IL-10, that are able to polarize emerging T-cell responses. Adding to the complexity, it is to date not clear whether all forms of activation of DC necessarily result in increased immunogenicity. Furthermore, DC can produce different cytokines in response to different activating stimuli [64]. An example is shown by the observation that murine DCs phagocytosing either yeast or hyphae of *Candida albicans* produce either IL-12 or IL-4, and *in vivo* drive either Th1 or Th2 differentiation, respectively [65]. Therefore DC and macrophages are important at the interface in bridging the innate and adaptive immune system.

11.6
Dendritic Cells as Sensors of Infection

The immune system has developed mechanisms to detect and initiate responses to a continual barrage of immunological challenges. Dendritic cells play a major role as immunosurveillance agents. To accomplish this function, DC are equipped with highly efficient mechanisms to detect pathogens, to capture, process and present antigens, and to initiate T-cell responses. The recognition of molecular signatures of potential pathogens, in DC, is accomplished by a membrane receptor of the Toll-like family (TLRs) which activates dendritic cells, leading to the initiation of adaptive immunity. TLR signaling in DC causes an increase in the display of MHC peptide ligands for T-cell recognition, upregulation of co-stimulatory molecules important for T-cell clonal expansion and secretion of immunomodulatory cytokines, which direct T-cell differentiation into effectors. Remarkably, ligation of distinct TLRs can trigger differential cytokine production in a single DC type or result in different cytokines in distinct DC sub-types. Studying the complexity of DC responses to TLR ligands illuminates the link between innate recognition and adaptive immunity, paving the way for improved vaccines and strategies to induce tolerance to autoantigens or allograft.

DC comprise distinct subsets: human blood contains at least two distinct DC types, the myeloid DCs CD11c+ and the plasmacytoid DC (PDC), as well as the monocyte precursors of Mon-DC [66]. Unlike CD11c+ DC and Mon-DC, PDC may have primarily an innate role in regulating antiviral responses although they can also act as antigen-presenting cell. Mon-DC, monocytes and neutrophils, expressed mRNA for TLRs 1, 2, 4 and 5 but only Mon-DC expressed TLR3 message [67]. Similarly, subsequent studies reported a decrease in expression of TLRs 1, 2, 4, 5 and 8 but an increase in TLR3 during monocyte differentiation into DC [68, 69]. In contrast, human PDC do not express message for TLRs 2, 3, 4, 5 and 8 and are unre-

sponsive to peptidoglycan, lipoteichoic acid, poly I:C and LPS [70]. PDC express TLR9, which is not found in monocytes, granulocytes, Mon-DC and CD11c+ DC.

The situation is different in mouse: mouse spleen PDC express TLR7 and TLR9 but, in contrast to human, they also express mRNA for most other TLRs [71]. TLR9 is expressed by both murine plasmacytoid and nonplasmacytoid DC. Thus, discussion of TLR repertoires should be restricted to particular cases where a given DC subset does not express detectable mRNA or respond to ligands for a particular TLR. We can use information about TLR repertoires and DC subset biology to predict some of the functions of TLRs in the immune system.

11.7 DC Transcriptional Profile Induced by Pathogen Teaches the Dynamic of the Interactions: DC Transcriptome; Core and Specific Responses

DC have a crucial role in linking the class of immune response to the invading pathogen through the differential expression of T-cell polarizing signals upon the ligation of selective pattern recognition receptors. The detailed mechanisms are still unknown. It is emerging that Th1 responses are initiated by intracellular TLR (TLR3, 7, 8 and 9) on DC, resulting in high expression of IL-12 gene family.

The microarray analysis has been used to study the DC transcriptome upon infection, by comparing the gene expression responses of dendritic cells to a bacterium (*E. coli*), a virus (influenza A), and a fungus (*C. albicans*) [72]. In this work a core of 166 genes that were induced by each organism in dendritic cells was described.

The expression pattern of these genes indicated the sequence of events and coordination of pathways involved in immune responses.

Genes whose transcripts declined soon after pathogen contact include those involved in pathogen recognition and phagocytosis. Also at this stage there was upregulation of genes expressing cytokines, chemokines, and immune cell receptors which allowed recruitment of other innate immune cells to the site of infection and genes modulating the cytoskeleton, which the authors postulated may be involved in DC migration. By 12 h after infection there was increased expression of transcription factors and signalling molecules involved in lymphoid tissue regulation, antigen processing, and presentation. By 18 h there was upregulation of chemokine receptor expression, thought to be related to the migration of DC to lymph nodes. In the time period frame analyzed there was a sustained upregulation of production of reactive oxygen species, suggesting that there was continued killing of organisms by dendritic cells. This common core response was independent of pathogen characteristics and occurred in a coordinated fashion modulating innate and adaptive responses.

11.8
DC and Shistosoma Mansoni Specific Signature

Expression analyses have showed that after microbial interaction, DC undergo a multistep maturation process [24] and acquire specific immune functions, depending on the type of microbe they have encountered.

We have defined in detailed the transcriptome induced in murine dendritic cells by different pathogens such as *Shistosoma Mansoni* and *Leishmania Mexicana*. The data clearly demonstrate that individual parasites induce both common and individual regulatory networks within the cell. This suggests a mechanism whereby host–pathogen interaction is translated into an appropriate host inflammatory response.

S. Mansoni is a helminth parasite, which has a complex life cycle that is initiated by the transcutaneous penetration of the larvae followed by its rapid transformation into schistosomula (SLA) [73, 74]. Once in the skin, SLA closely interact with immunocompetent cells, including dendritic cells, to manipulate the host immune response [75, 76]. SLA then begin a long vascular journey to reach the intrahepatic venous system, where they mature into adult male and egg-producing female worms. Eggs that accumulate in the liver, spleen, and lungs induce inflammation and an intense granulomatous hypersensitivity reaction [77].

We have investigated DC–schistosome interactions using a genome-wide expression study. We have used a near-homogeneous source of mouse DC, the well-defined, long term D1 splenic population [78]. The kinetic global gene expression analysis of mouse DC stimulated with eggs or SLA indicated that genes encoding inflammatory cytokines, chemokines, and IFN-inducible proteins were oppositely regulated by the two stimuli (Fig. 11.3). Interestingly, eggs, but not SLA, induced the expression of IFN-β that efficiently triggered the type I IFN receptor (IFNAR) expressed on DC, causing phosphorylation of STAT-1 with consequent upregulation of IFN-induced inflammatory products.

Clustering techniques applied to 283 differentially expressed genes distinguished the two stimuli from different points of view (Fig. 11.4). The egg time-course experiment was compatible with a progressive cell differentiation process, such as maturation, whereas observations from SLA-stimulated DC samples suggested the occurrence of a stable blocking event within the first 4 h. Moreover, eggs modulated different amounts and subsets of genes in comparison with SLA, indicating that the two developmental stages of *S. mansoni* affected distinct intracellular pathways in DC possibly by triggering specific receptors. The egg stage sustains the maximization of Ag presentation efficiency in DC by inducing the upregulation of H-2M, which plays a crucial role in the peptide loading of MHC class II molecules [79] and of the co-stimulatory molecules CD40 and ICAM-1. Cathepsins D and L, which are believed to remove invariant chain from its complex with MHC class II molecules [80], are downregulated by SLA, but are not modulated by eggs, suggesting a reduction in the Ag processing capacity exerted by the larval stage on DC. Moreover, the egg stage induced the expression of pro-inflammatory cytokine transcripts, such as TNF-α, and chemokines, such as IP-10 (CXCL10), monocyte chemoattractant pro-

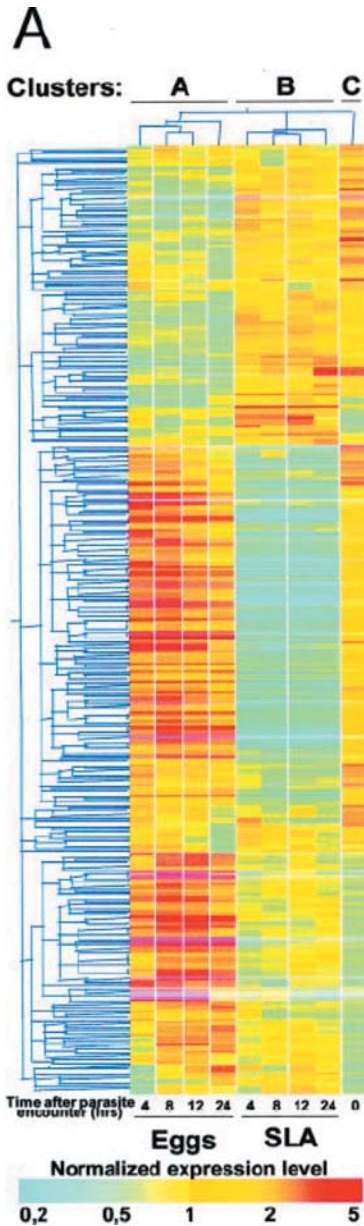


Fig. 11.3 Expression profile clustering of 283 genes differentially expressed during DC–schistosome interaction. Two-way hierarchical clustering of gene expression profiles measured in time-course experiments; normalized expression levels relative to median are displayed in yellow (median expression), red (increased expression), or cyan (decreased expression) according to the color bar.

tein-5 (CCL12), MIP-1α (CCL3), MIP-1α (CCL4), MIP-1γ (CCL9), and MIP-2 (CXCL2), that are known to collectively attract granulocytes, immature DC, NK cells, and activated T cells [81]. *S. mansoni* eggs, but not SLA, induced the production of high amounts of IL-2, which could be important for DC-mediated activation of NK cells or NKT cells as well as for priming naïve T cells.

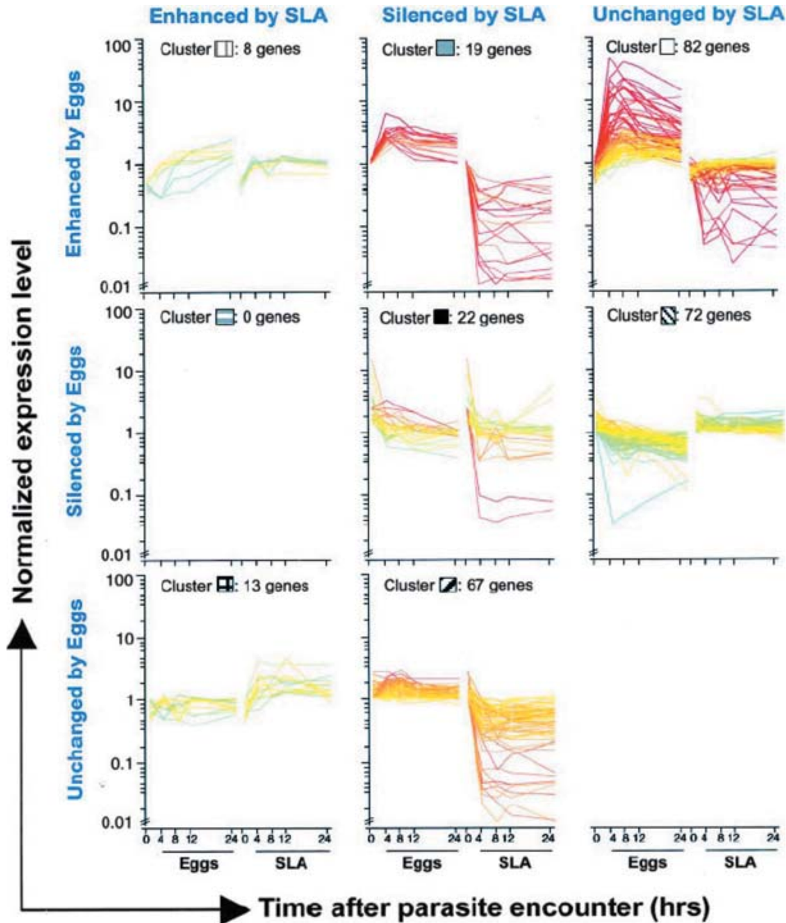


Fig. 11.4 Expression profile clustering of 283 genes differentially expressed during DC–schistosome interaction. Supervised clustering of kinetic gene expression profiles. Each panel groups genes that share same transcriptional response (enhanced, silenced or unchanged) relative to the two developmental stages of the parasite. Each line represents the expression profile of a particular gene and is colored according to its normalized expression level 4 h after encountering *S. mansoni* eggs.

Mouse myeloid DC, in response to helminth eggs, activate a strong interferon response compared to SLA (Fig. 11.5). We have observed that the DC-derived IFN-β molecule efficiently triggered the IFNAR expressed on DC, thus providing an autocrine and/or paracrine stimulation mechanism. Therefore, our data indicate myeloid DC as one possible mediator of type I IFN signaling as well as one plausible source of IP-10 and MIP-1α production also in response to helminth infections. The comparative gene expression analysis revealed two different DC global transcriptional modifications induced by either Schistosoma eggs or SLA, consis-

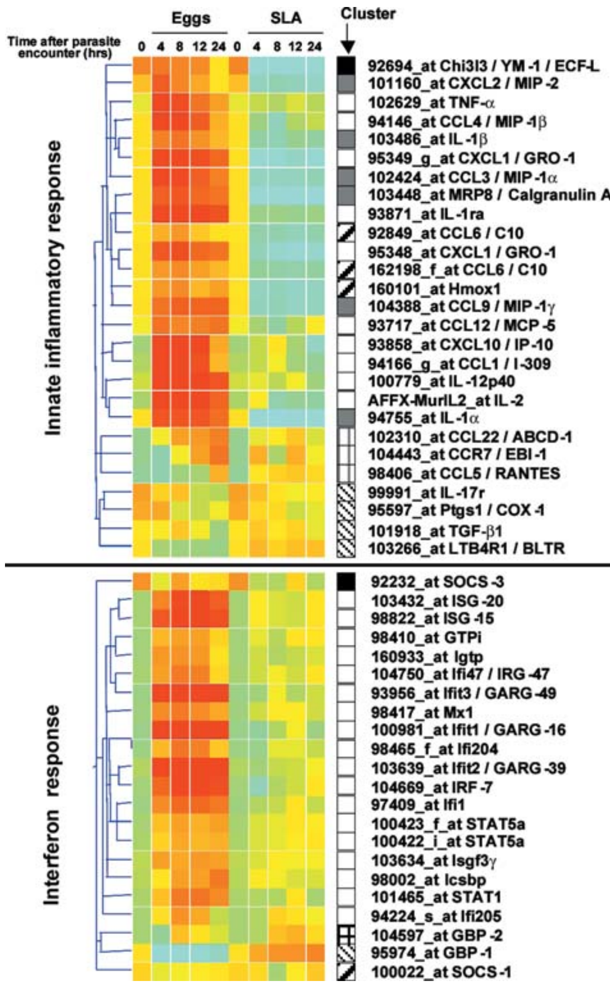


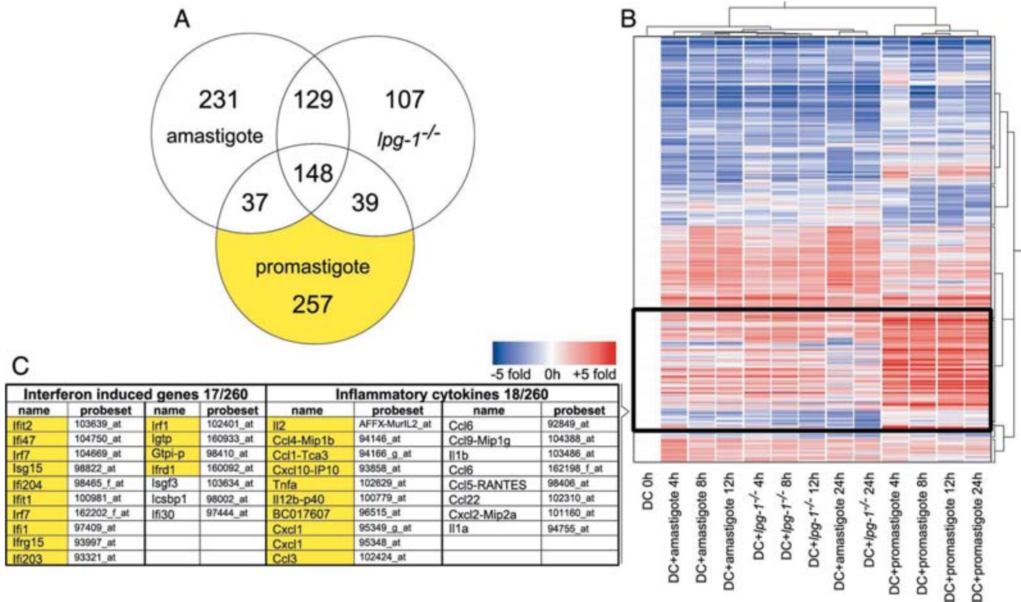
Fig. 11.5 Functional classification of differentially expressed genes. Each colored box represents the normalized expression level of a given gene in a particular experimental condition and is colored according to the color bar. Interferon-induced genes are shown.

tent with the different responses induced *in vivo* by these two parasite stages. Taken as a whole, our observations have provided new molecular insights into the host–parasite interaction established in the course of schistosomiasis leading to the identification of a type I IFN-dependent mechanism by which DC may amplify inflammatory reactions in response to helminth infection.

11.9
DC and Leishmania Mexicana: Molecular Profile of the Interaction

A molecular signature was generated by DC after interaction with the protozoan parasite *L. mexicana*. Parasite infection is initiated by the transfer of insect vector-borne promastigotes. These are taken up by phagocytic cells and transformed into the obligate intracellular amastigotes, which dwell within vacuoles with endosomal-early-lysosomal characteristics [82, 83]. Uncontrolled replication of the parasites causes disease; to resolve the infection the host generates leishmanicidal mechanisms consisting primarily of the activation of phagocytes through cytokines, e.g. IFN-γ and TNF-α [84].

DC activation was shown upon interaction with *L. mexicana* promastigotes but not with *L. mexicana* amastigotes, as indicated by MHC II, CD86, CD54 expression



Interferon induced genes 17/260				Inflammatory cytokines 18/260			
name	probeset	name	probeset	name	probeset	name	probeset
Ifit2	103639_at	Irf1	102401_at	Il2	AFFX-MurIL2_at	Ccl6	92849_at
Ifi47	104750_at	Igtp	160933_at	Ccl4-Mip1b	94146_at	Ccl9-Mip1g	104388_at
Irf7	104669_at	Gtpi-p	98410_at	Ccl1-Tca3	94166_g_at	Il1b	103486_at
Isg15	98822_at	Ifrd1	160092_at	Cxcl10-IP10	93858_at	Ccl6	162198_f_at
Ifi204	98465_f_at	Isgf3	103634_at	Tnfa	102629_at	Ccl5-RANTES	98406_at
Ifit1	100981_at	Icsbp1	98002_at	Il12b-p40	100779_at	Ccl22	102310_at
Irf7	162202_f_at	Ifi30	97444_at	BC017607	96515_at	Cxcl2-Mip2a	101160_at
Ifi1	97409_at			Cxcl1	95349_g_at	Il1a	94755_at
Ifrg15	93997_at			Cxcl1	95348_at		
Ifi203	93321_at			Ccl3	102424_at		

Fig. 11.6 *L. mexicana* promastigotes expressing LPG trigger a pro-inflammatory transcriptional response in D1 cells. (A) A total of 948 differentially expressed mRNAwere organized according to common or specific modulation in D1 cells infected with *L. mexicana* amastigotes or wild-type or lpg1–/– mutant promastigotes (lpg1–/–). (B) Hierarchical clustering according to samples (vertical) and gene expression profiles (horizontal). Mean gene expression values are shown, and gene signals are divided by their 0-h value. Red and blue colors indicate up- or downregulation, respectively, in comparison to 0-h time point values (white). A cluster of 260 genes that contains all differentially expressed genes belonging to interferon-induced genes ($n = 17$) and inflammatory cytokine genes ($n = 18$) is boxed. (C) These genes, which belong to the promastigote-specific group of the Venn diagram (A), are color-coded in yellow.

and IL-12p40 synthesis. The transformation from the extracellular promastigote stage to the intracellular amastigote stage of Leishmania is associated with a dramatic change in the molecular composition of the parasite surface. The surface of promastigotes is almost entirely coated by the lipid-anchored glycan, lipophosphoglycan (LPG). In contrast, in amastigotes of most species, LPG expression is severely downregulated [85] or completely undetectable [86]. Instead, the cell surface of amastigotes is dominated by small glycolipids, mostly glycoinositolphospholipids, and is poor in proteins.

We have investigated the nature of the *L. mexicana* promastigote-derived DC activating signal: again we employed comparative global transcriptional analysis to study the response of DC cell line D1 to *L. mexicana* promastigotes and amastigotes and to lpg1–/– promastigotes (Fig. 11.6). Only LPG expressing *L. mexicana* promastigotes induce a pro-inflammatory programme in DC and suggest that the amastigotes have evolved to downregulate LPG expression in the mammalian host in order to achieve immune escape.

We show that DC mount a pro-inflammatory transcriptional response to *L. mexicana* parasites that depends on LPG expression. Both hierarchical clustering and principal component analysis (PCA) allowed us to visualize the distinct impact of pro-inflammatory genes within the global effects of *L. mexicana* infection on DC (Fig. 11.7). The significant role of LPG expression for this response was further

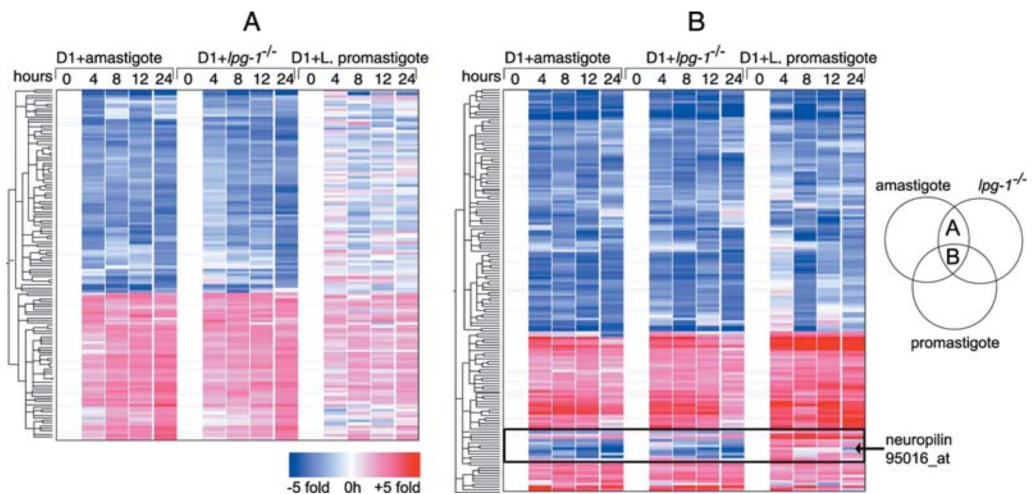


Fig. 11.7 Hierarchical clustering of commonly modulated mRNA reveals an LPG-induced signature in D1 cells. The expression profiles of 129 genes modulated in D1 cells after infection with either amastigotes or lpg1–/– mutants is represented in (A), whereas the expression profiles of 148 genes modulated by all three parasite forms is represented in (B) Time course profiles are shown horizontally. The black box highlights a new part of the LPG signature, and neuropilin is indicated (see text for details). Red and blue colors indicate up- and downregulation, respectively, in comparison to 0-h time point values (white).

supported by the finding that infection with mutant parasites, where LPG-expression was restored, reproduced the expression pattern of these signature genes. Hierarchical clustering of genes classified according to Venn diagram allowed the identification of additional genes differentially modulated by LPG-positive and LPG-negative parasite forms. Overall, the response to LPG expressing parasites is consistent with the biological role of DC, i.e. their migration to local lymphoid centers, increase in Ag presentation capacity and control of immunity in the early phase via the production of key cytokines following the recognition of a pathogen.

LPG downregulation in the amastigote stage may be the result of evolutionary adaptation. However, it also became clear during this study that infection with *L. mexicana* amastigotes is not a silent process but results in a complex response in DC. Apart from not triggering a pro-inflammatory response this may have additional functional consequences which need to be addressed in future studies.

11.10 Conclusions

High density DNA microarray analysis of host gene expression provides a powerful method of examining microbial pathogens from a novel perspective. The ability to survey the responses of a large subset of the host genome, and to find patterns among the profiles from many different microorganisms and hosts, allows fundamental questions to be addressed about the basis of pathogen recognition, the features of the interaction between host and pathogen and the mechanisms of host defense and microbial virulence. This emerging technology will illuminate our understanding of the molecular basis of the host–pathogen interaction. The biological insights thus gained are likely to lead to major shifts in our approach to the diagnosis, treatment, assessment of prognosis, and prevention in many types of infectious diseases within a decade.

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III
Dendritic Cells in Secondary Lymphoid Organs

12
Dendritic Cell Subtypes

Ken Shortman and Jóse A. Villadangos

12.1
Introduction

The identification of dendritic cells (DC) by Steinman and Cohn [1] and the elucidation of their crucial role as antigen-presenting cells [2] was a major advance in immunology. The realization that the Langerhans cells from the epidermis were functionally related to the DC found in the spleen then provided the following unified model of DC life history [2–4]. DC begin in an immature form as antigen-sampling sentinels at peripheral sites. On encountering microbial products or other "danger" signals they migrate to lymphoid organs and mature into an antigen-presenting form now capable of activating T cells. The subsequent development of culture systems for the production of DC from monocytes or from myeloid precursors, under the influence of granulocyte-macrophage stimulating factor (GM-CSF), then provided a readily available model for detailed study [5–8]. In the face of such a unifying paradigm with an experimentally accessible culture model, the finding of discrete DC subtypes within lymphoid tissue was initially considered a most unnecessary complication. The search for DC subtypes seemed akin to stamp collecting: great fun for those so inclined, but of little help in understanding the basics of the immune system.

As further information on DC subtypes accumulated, this perception has changed. All the DC subtypes do share the basic DC functions. They are able to take up antigens and process them for MHC presentation. After appropriate activation they assume a dendritic form and are able to activate T cells. This is part of the definition of a DC. However, we are now aware of many specializations in the detailed functions of DC, such as those summarized in Table 12.1, and these provide some biological rationale for the existence of multiple DC subtypes.

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Tab. 12.1 Some functional differences amongst spleen DC subtypes

	References	*pDC*	*CD8⁺ cDC*	*DN cDC*	*CD4⁺ cDC*
Surface markers	14	$CD11C^{lo}$ $CD45RA^{hi}$ $CD11b^{-}CD205^{-}$ $CD4^{\pm}$ $CD8^{\pm}$	$CD11c^{hi}$ $CD45RA^{-}$ $CD11b^{-}CD205^{+}$ $CD4^{-}8^{+}$	$CD11c^{hi}$ $CD45RA^{-}$ $CD11b^{+}CD205^{-}$ $CD4^{-}8^{-}$	$CD11c^{hi}$ $CD45RA^{-}$ $CD11b^{+}CD205^{-}$ $CD4^{+}8^{-}$
TLR distribution	84, 85	$2^{+}3^{-}4^{-}$ $5^{\pm}7^{+}9^{+}$	$2^{+}3^{+}4^{+}$ $5^{-}7^{-}9^{+}$	$2^{+}3^{\pm}4^{+}$ $5^{+}7^{+}9^{+}$	$2^{+}3^{-}4^{+}$ $5^{+}7^{+}9^{+}$
Constitutive crosspresentation of exogenous antigens on MHC I	71, 86	–	+++	±	–
Protease Inhibitor cystatin C	73	±	+++	±	±
Production of IFNα (CpG or influenza virus stimulation)	28, 29	+++	±	–	–
Production of IL–12p70 (cytokine plus TLR9 stimulation)	29, 87–89	+	+++	±	–
Production of IFNγ (IL–12 & IL–18 stimulation)	90	–	–	+	–
Production of inflammatory chemokines	85				
Steady–state		–	–	±	+
Activated		+++	++	+++	+++
Induction of Th1 responses	89, 91, 92	–	+++	±	±

DC are localized in many different body sites, one major reason for the existence of different DC subtypes. A differing localization requires differences in chemokine receptors, addressins, adhesion molecules and other surface components; it is no surprise that a Langerhans cell serving as a sentry in the skin epidermis differs from an interstitial DC within an internal organ. In addition, DC must recognize and respond to a wide range of potential pathogens. There are differences between DC subtypes in the distribution of Toll-like receptors (TLR), lectins and other microbial product receptors, as well as differences in antigen uptake and processing. Furthermore, DC must initiate different types of T-cell immune responses to dif-

ferent pathogens, the Th1 versus Th2 responses being the classic example. This is in part determined by the cytokines and chemokines produced by DC, and the different DC subtypes have different potentials in this respect. Finally DC are involved not only in inducing immunity, but also in maintaining tolerance to self components. Although these two roles can be assigned to different stages in the development of a single DC subtype, there is likely to be further specialization at this level. There is no lack of possible reasons for the multiplicity of DC subtypes, and a clear need to understand their development and functional potential.

We consider it important to avoid unnecessarily complicating an already complex field by considering every DC differing in some surface marker as a distinct subtype. Differences in developmental or activation states lead to differences in surface markers, especially in MHC and co-stimulatory molecule levels. We will define two DC differing in surface markers as being of different subtypes if one cannot be transformed into the other, although both may well have a common precursor not too far back in development. This is deliberately to face the most controversial aspect of the field, since in dealing with these responsive and plastic cells, the developmental relationships are not always clear. The DC subtypes we discuss in this chapter appear to fit our definition, although critical experimental testing has not always been applied. Most of this chapter will concern the DC of mice, where subtype segregation has been studied in most detail; however we will briefly compare mouse with human DC.

12.2 DC Surface Antigen Heterogeneity and the Recognition of DC Subtypes

The first flow cytometric analyses of DC isolated from lymphoid tissues revealed heterogeneity in surface staining for many markers commonly used for segregating lymphoid or myeloid cells, including CD4, CD8, Fc receptors and integrins [9]. The concept that these surface markers could be used to segregate discrete DC subtypes developed when clear discontinuities in marker levels were observed, and when cross-correlation studies revealed that several different markers produced the same subset groupings [10–16]. Very few markers have proved to be DC subtype specific, or even DC specific, so multiparameter analysis using several markers is usually required to distinguish DC subtypes. The markers originally used to discriminate DC subsets provided little guidance to function. However separate studies revealed some striking differences in the immunological properties of these DC subtypes (Table 12.1). Finally, the concept arose that many of these subtypes were products of separate sub-lineages or branches in the DC developmental stream [17]. This was based on three lines of evidence. This first was that one DC subtype did not transform into another on activation or maturation in culture, or on transfer to recipient mice. The second was the differences in the kinetics of DC generation and turnover as measured by the uptake of a DNA precursor. The third was the dependence on different transcription factors for the development of the different DC subtypes.

One potential hazard in distinguishing DC subtypes based on surface marker staining is the propensity of DC to acquire surface antigens from associated T cells. Thus thymic DC acquire CD8αβ, CD4 and Thy1 from thymocytes, although they do not seem to acquire CD3, T-cell antigen receptor or CD45 in this way [14]. The level of staining from such pickup of antigens is usually moderate rather than high. There are several ways to test if the staining obtained represents authentic expression of the marker by the DC themselves. One way is to check that the DC express the appropriate mRNA, as well as staining for the surface protein. Another is to make bone-marrow chimeras using a mix of bone-marrow cells from wild-type mice and from mice deficient in the gene coding the marker protein; any staining for the marker on the surface of the cells genetically unable to synthesize it, proves that it was derived from the other cells [14]. Finally, in the case of CD8 where the DC form is the CD8αα homodimer rather than the CDαβ heterodimer as on T cells, any staining for CD8β is an indicator of marker pickup from T cells. The DC surface markers we describe appear to be products of the DC themselves.

12.3
DC Subtypes in Steady-state versus Infected Mice

Most of this chapter will be concerned with the DC found in the lymphoid organs of clean laboratory mice, the steady-state situation where the DC network is more involved with maintaining self-tolerance than responding to infections. This is an artificial situation compared to normal human populations. It must be emphasized that DC can change radically when stimulated by infection or inflammation *in vivo*, or even by simple incubation in culture. As well as the expected upregulation of markers of DC "maturation" (MHC II, B7 family co-stimulatory molecules) [11, 18–20], markers used for subset discrimination can change so radically that the DC move outside the normal subset sorting gates. Thus even overnight culture in normal media can result in a marked drop in CD4 levels, and a significant drop in CD8 [21]. In contrast the surface levels of DEC-205 and CD11b generally increase [11]. Although a differential between DC subtypes in expression of DEC-205 is usually maintained, some DC subtypes previously CD205 "negative" may now stain as brightly as the "positive" cells. Even more radical changes in DC subset distribution can occur *in vivo*. There is a transient loss of some DC after microbial stimulation [19, 21]. Finally, novel DC forms not well represented in steady-state may appear after infection or inflammation. The monocyte-derived DC, so readily generated in culture, may only be a minor DC component *in vivo*, except during inflammatory responses when GM-CSF is elevated [22–24]. Another example is the changes in plasmacytoid DC (pDC), normally a round, nondendritic form with a characteristic surface antigen phenotype distinguishing it from conventional DC (cDC). Stimulation in culture, or by viral infection *in vivo*, can transform pDC into a dendritic form with many of the markers seen on cDC, but differing in others such as CD205 and CD69, so demonstrating it is a novel DC form not apparent in steady-state mice [23, 25–29].

12.4 Extraction and Enrichment of DC from Lymphoid Tissue

Although many DC are released by mechanical disruption of lymphoid tissue, efficient recovery of all DC subtypes requires mild digestion with collagenase [10]. Digestion at room temperature is usually sufficient and avoids the DC activation which is initiated at 37 °C [18, 20]. Subsequent chelation of Ca^{2+} and Mg^{2+} with EDTA helps release of DC from any complexes with T cells [10]. Although direct staining and flow-cytometric analysis of DC in such a suspension is possible, the low DC frequency and the overlap of marker expression with other cells usually means significant contaminants are present in the DC gate. Pre-enrichment prior to analysis or sorting can overcome this. The simplest procedure is selection of the 5% lightest density cells, which includes all the DC. Immunomagnetic bead depletion of non-DC can further enrich the DC, although here care must be taken not to selectively deplete particular DC subtypes. Thus DC subsets will be lost if T cells are depleted using anti-CD4 or anti-CD8, but not using antibodies against CD3 or the T-cell receptor. The pDC subtype will be lost if anti-B220 is used to deplete B cells, but not if anti-CD19 is used. Potentially pDC might be lost using some anti-Ly6G antibodies which cross-react with Ly6C (such as RB6 8C5) to deplete granulocytes; although we have not found this a problem, it is safer to use more specific anti-Ly6G mAb (such as IA8). An alternative strategy for enrichment and purification is to use immunomagnetic bead procedures to positively select for DC based on CD11c expression, although this alone will not give the level of purity obtained by fluorescence activated cell sorting of enriched DC preparations.

12.5 Plasmacytoid versus Conventional DC

A useful primary division of the DC in all lymphoid tissues is into the plasmacytoid DC (pDC) versus the more conventional DC (cDC) subtypes. These are often referred to as "lymphoid" DC and "myeloid" DC respectively, unfortunate terms since they suggest a lymphoid precursor versus a myeloid precursor origin, in conflict with current evidence [30, 31]. pDC might be best termed pre-dendritic cells, since until they are appropriately activated they do not have a dendritic morphology and they lack the antigen processing properties and the capacity to activate T-cells of the conventional DC. Their relatively long lifespan suggests that this activation to a different form does not happen frequently in steady-state mice, and it normally requires a microbial stimulus [29]. pDC have a capacity to rapidly produce large amounts of class I interferons in response to viral or microbial stimuli, and so are also termed the natural interferon-producing cells [32]. pDC are present in all lymphoid organs. Some of their distinctive properties are presented in Table 12.1. Their functional properties are considered in detail elsewhere in this volume; in this chapter we will focus on the phenotypic markers that set them apart from cDC.

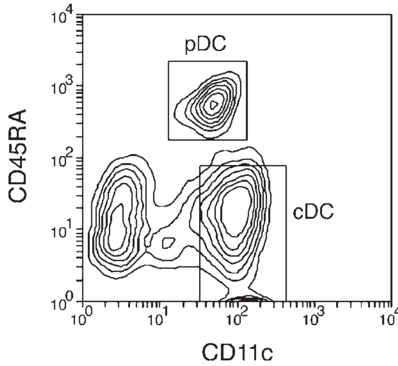

Fig. 12.1 FACS analysis of expression of CD11c and CD45RA in a thymic DC preparation carried out as described [29]. The squares indicate the regions containing the cDC and the pDC. The CD11c⁻CD45RA⁻ cells are contaminants in the preparation.

pDC express CD11c, but generally at lower levels than cDC, and are readily distinguished from cDC by expressing CD45R (B220) and CD45RA [26–29]. Sorting an enriched DC preparation on the basis of these markers thus provides a good purification and separation of both pDC and cDC (Fig. 12.1). pDC are $CD11b^-$ and $CD205^-$, which also distinguishes them from particular cDC subtypes. Recently new markers and mAb have been developed that are relatively specific for pDC [33, 34], simplifying their identification and study.

pDC show heterogeneity of expression of several surface markers. They may be subdivided on the basis of Ly6C or Ly49Q expression [35, 36]. However, it is likely that these represent different developmental states of pDC, with the $Ly6C^-$ and $Ly49Q^-$ components being less mature. pDC from lymphoid organs also express various levels of CD4 and CD8α, whereas those in bone-marrow and blood are $CD4^-8^-$ [37]. However, in contrast to the situation with cDC, the $CD4^+$ and $CD8\alpha^+$ subsets appear to be different developmental states of the one pDC lineage [29]. pDC development may eventually be shown to include sub-lineages leading to functionally distinct pDC subtypes, but at present the data fits best into a single lineage, single subtype model.

In contrast to pDC, the cDC may be separated into discrete subtypes which differ in functional properties (Table 12.1) and which appear to represent distinct developmental end products or sub-lineages. The cDC subtype distribution differs between different lymphoid organs.

12.6 Spleen DC Subtypes

Mouse spleen is a commonly used source of DC and the splenic DC subpopulations are amongst the best characterized. The DC of spleen, as well as functioning to maintain self-tolerance, serve as sentinels monitoring the blood stream [38–40]. Rather than arriving through the lymph, splenic DC derive from blood, arriving as immature DC or DC precursors, with the spleen itself serving to generate the final DC products or to expand their number. Splenic DC consist of around

20% pDC, 80% cDC. The cDC may be separated into three distinct and dominant subtypes [14]. Less frequent and less distinct DC subsets are also sometimes evident [41].

Segregation of the three major subtypes has been achieved by multiparameter analyses based on differences in expression of CD8α, CD4, CD11b (Mac-1) and CD205 (DEC-205). Other markers, such as CD24 (heat stable antigen) and Sirp α, can help define the same subtypes. Although the level of staining for these key markers is usually designated "negative" or "positive, "negative" for CD11b and CD205 really means low expression, readily distinguished from and not continuous with the high expression profile of the "positive" population. The segregation is often only clear-cut in the steady-state DC population, since, as mentioned above, the expression of CD4, CD8, CD11b, CD205 and even CD11c may change on DC activation. An example of splenic cDC segregation based on CD4 and CD8 expression is shown in Fig. 12.2. Both these markers have been shown to be products of the DC themselves, rather than originating from T cells [14]. The CD8 is in the form of an αα homodimer rather than the αβ heterodimer of T cells [10]. Despite the relatively high expression of CD4 and CD8α, the same DC subtypes are still present and may be detected using alternative markers in mice lacking functional CD4 or CD8α genes. To date no functional DC defects have been reported in the absence of CD4 and CD8.

About half the cDC of the spleen are $CD4^{+}8^{-}$ (termed $CD4^{+}$ cDC), and are also $CD11b^{+}$ $CD205^{-}$ $CD24^{-}$ Sirp α^{+}. Another subtype is $CD4^{-}8^{-}$ (termed "double negative" or DN cDC), and is likewise $CD11b^{+}$ $CD205^{-}$ $CD24^{-}$ Sirp α^{+}. Both the CD4 cDC and the DN cDC are found mainly in the marginal zones of the spleen in steady-state mice, although they move to the T-cell areas on activation [16, 19, 42]. These two populations, being similar in surface phenotype and many functional properties, are often pooled together as $CD8^{-}$ DC. However they do display some functional differences (Table 12.1). Their gene expression profiles show extensive overlap [43], but there are differences, notably in a group of genes expressed by the DN but not the CD4 cDC (Mireille Lahoud and Ken Shortman, unpublished results). Many of these features would be compatible with a model where $CD4^{+}$ cDC is a less mature form and a precursor of the DN cDC subtype. The drop in CD4 levels on maturation of the $CD4^{+}$ cDC in culture lends support to this view [14]. How-

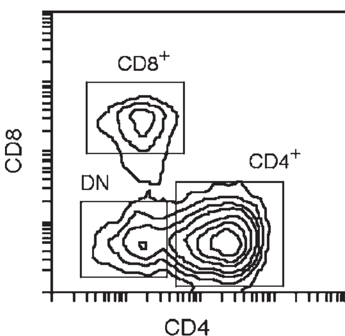

Fig. 12.2 FACS analysis of expression of CD4 and CD8 in splenic cDC. The squares indicate the regions containing the $CD4^{+}$, $CD8^{+}$ and double negative (DN) DC populations.

ever, some experimental data argues against this. Firstly, the MHC II and co-stimulatory surface expression by the DN and $CD4^+$ cDC are similar, rather than the DN cDC appearing more “mature” [14, 16]. Secondly, BrdU labeling studies suggest an independent origin, with the CD4 cDC being labeled later rather than earlier than the DN cDC [21, 44]. Finally, continuous anti-CD4 depletion of the CD4 cDC population does not affect the level of the other spleen cDC subtypes [21], indicating that it cannot be an obligatory precursor of the other subtypes. Tentatively these two $CD8^-$ cDC are considered as separate if closely related subtypes.

The 23% of spleen cDC which are $CD4^-8^+$ ($CD8^+$ cDC), represent a more distinct subtype. These DC are $CD11b^-$ $CD205^+$ $CD24^+$ Sirp α^-. In contrast to the $CD8^-$ cDC subtypes, many are located in the T-cell zones of steady-state mice [19, 42]. The gene expression profile of the $CD8^+$ cDC shows many differences from the $CD8^-$ populations [43]. There is evidence from culture studies that CD8α is induced on this DC subtype during the latter stages of its development [45] and evidence that activation can induce CD8α expression, at least at low levels, on other DC lineages [46, 47]. This led to the concept that CD8α is an “activation marker”, and that splenic $CD8^+$ cDC could be a more mature form of $CD8^-$ cDC. However, there is substantial evidence against this. Firstly, although the levels of MHC II and some co-stimulator molecules may be slightly higher on $CD8^+$ DC than on $CD8^-$ DC, these differences in surface expression are very slight and not compatible with a general activation event [40]. Secondly, $CD8^-$ cDC do not become $CD8^+$ on activation in culture [14], nor in our experience, on transfer to recipient mice [48]. Thirdly, depletion of CD4 DC by continuous anti-CD4 treatment does not reduce the level of $CD8^+$ cDC, eliminating the major $CD8^-$ cDC subset as a direct precursor [21]. Fourthly, kinetic studies of continuous BrdU uptake suggest an independent origin for $CD8^+$ cDC, with an initial labeling rate faster than that of the $CD8^-$ cDC rather than lagging behind [21]. Finally, studies using genetically manipulated mice indicate that development of $CD8^+$ cDC, but not of $CD8^-$ cDC is dependent on the transcription factor IRF 8 [49, 50] whereas development of $CD8^-$ cDC but not $CD8^+$ cDC is dependent on the transcription factor IRF 4 [51]. Together this suggests that $CD8^+$ cDC represents a distinct subtype and the product of a separate sub-lineage, rather than developing from $CD8^-$ cDC. However, it must be emphasized that all these DC subtypes could derive from a common precursor and represent very late branch points in the developmental pathway.

12.7
Lymph Node DC Subtypes

Lymph nodes (LN) contain examples of the same DC subtypes found in spleen, namely the pDC, $CD8^+$ cDC and $CD8^-$ cDC, with the difference that the proportion of $CD4^+$ cells amongst the $CD8^-$ cDC is much lower [15]. Pickup of some CD4 from T cells complicates analysis of CD4 expression by LN DC. It is assumed, by analogy with spleen, that these DC or their immediate precursors arrive in LN via the blood. This seems especially likely for the pDC which exhibit some of the migratory char-

acteristics of lymphocytes, but remains to be established for the "spleen-like" cDC subtypes. However, LN DC differ in an important way from those in spleen, since they include as additional components migratory DC which arrive via the lymph. There is a continuous input of DC into LN from lymph even in steady state [12, 21, 44, 52–55], in the absence of obvious "danger signals", although this rate increases with infection or inflammation [56–59]. Furthermore, the subtypes of migratory DC differ depending on the tissue being drained, so different LN contain a different spectrum of DC subtypes. However, the migratory cDC do exhibit some common features. As discussed below, they generally have a "mature" phenotype with higher surface levels of MHC II and co-stimulatory molecules. They stain at moderate to high levels for CD205. Although some express low or even intermediate levels of CD8α, they separate in a $CD205^+$, $CD8^-$-to-$CD8^{low}$ gate that is quite distinct from the $CD8\alpha^{hi}$ $CD205^{hi}$ region that contains the $CD8^+$ DC population.

Mesenteric LN contains a population of $CD8^{low}$ $CD205^+$ $CD11b^+$ cDC not seen in significant numbers amongst spleen DC. These are assumed to be DC which have migrated from the gut, probably via Peyer's patches. Peyer's patches contain DC populations overlapping those found in mesenteric LN, but also contain a $CD8^-$ $CD11b^-$ subtype that is not clearly evident in most mesenteric LN DC in analyses to date [60]. It is possible that the migratory cDC in mouse mesenteric LN will prove to be heterogeneous when further analyzed. In the rat, MacPherson and colleagues have demonstrated two distinct subtypes of DC (or "veiled cells") in lymph draining the gut, one $CD4^-$ $OX41^-$ Sirp α^{lo} the other $CD4^+OX41^+Sirp\alpha^{hi}$ [61]. The former contains apoptotic fragments of gut epithelium, and is assumed to convey self-antigens for the maintenance of tolerance. Since there are differences between mouse and rat DC in the sets of markers currently used for DC subtype discrimination, the level of correspondence between the mouse and rat DC subtypes is not yet clear.

LN which drain the skin have a well established heterogeneity in their migratory cDC population, corresponding to DC derived from the epidermis – Langerhans cells – and DC derived from the dermis. These migratory cutaneous DC have been studied within the skin itself and as cells which migrate into the medium from cultured skin sheets. By labeling the skin with fluorescent tracker dyes, it has been possible to identify the skin derived DC in the draining LN [12, 15, 62, 63]. The cDC derived from the dermis have similar properties and may be equivalent to the migratory DC found in mesenteric LN. The cDC derived from the epidermis (Langerhans cells) tend to be larger, to have higher levels of co-stimulator molecules and a different pattern of integrins. They have higher levels of CD205 than most dermal-derived DC, which allows a partial but not complete separation of these two migratory cDC components in LN. Although they may express some CD8α after arrival in the LN and their level of CD205 is as high as on the $CD8\alpha^+$ cDC, these migratory DC remain clearly distinguishable from the latter based on the level of CD8α expression, as well as other markers. The Langerhans cells stand out from other DC populations by the expression of high levels of the lectin langerin [64]. This serves as the most useful single marker of this cDC subtype [58], although low levels of langerin are also expressed by $CD8^+$ cDC [15].

A third distinct type of migratory cDC has been detected in the LN draining lung, liver and kidney [65]. This subpopulation, which has antigen handling and presentation properties different from other migratory cDC, also has a unique pattern of surface markers, being $CD8^-$ $CD11b^-$ $CD205^+$ [65]. At face value this appears similar to a cDC population found in Peyer's patches [60], but further characterization would be needed to ascertain if they are equivalent. This novel migratory cDC has some of the features of the $CD8^+$ cDC subtype, suggesting it might be a precursor of the $CD8^+$ cDC population. However there is no evidence so far of such development or of upregulation of CD8α.

12.8 Thymic DC

The DC of the thymus are involved, along with thymic epithelial cells, in central tolerance of the developing T-cell repertoire. This focus on tolerance rather than immunity might be expected to require a particular spectrum of DC subtypes, and indeed thymic DC differ from those in spleen and LN. However evidence to date suggests it is more the developmental state of the T cells rather than the nature of the thymic DC which determines this balance towards tolerance [66]. The thymus, like other lymphoid tissues, contains pDC as well as cDC (Fig. 12.1). The cDC in the thymus are in the thymic medulla at the cortico–medullary junction, where they will encounter T cells that have already undergone positive selection and upregulated their T-cell antigen receptors. The pDC, although located mainly in the medulla, are also found in the cortex [36]. A substantial proportion of cDC are generated within the thymus itself, from the same lymphoid-restricted precursor population that produces T-cells; this may be part of the reason for the unique cDC subtype balance in the organ [67]. However, there is clear evidence that a proportion of thymic cDC are derived from the bloodstream, either as preformed cDC or as their immediate precursors [68]. The origin of mouse thymic pDC is not yet established.

The unique feature of thymic DC is the high proportion of $CD8^+$ DC amongst the cDC population; this varies from 70–90%, depending on mouse strain, in contrast to the 20% representation in peripheral lymphoid organs. These thymic $CD8^+$ cDC are $CD8\alpha\alpha^+$ $CD4^-$ $CD205^+$ $CD11b^-$, like those in spleen, although pickup of both CD4 and CD8αβ makes them difficult to analyze [14]. Thymic $CD8^+$ cDC share many functional properties of spleen $CD8^+$ cDC as listed in Table 12.1. However, they differ from peripheral cDC, since some express the early B-cell maker BP-1 and many bear D-J arrangements in their IgH genes [69]. This may reflect their origin from the early intrathymic T precursor population.

The thymus also contains a minor $CD8^-$ cDC population, difficult to analyze because of the pickup of CD8αβ from T cells. However it may be readily discriminated by staining for both CD8α and Sirp α, where it stands out as strongly Sirp α^+ (Mireille Lahoud and Ken Shortman, unpublished). This minor thymic cDC sub-

type is likely to be a component of the blood-derived rather than endogenously-generated thymic DC.

12.9 The Maturation State of Lymphoid Organ DC Subtypes

It was generally thought that any DC present within a lymphoid organ would be "mature", meaning unable to take up new antigenic material and equipped with the high surface levels of MHC II and co-stimulator molecules needed to initiate a T-cell immune response [70]. This followed the Langerhans cell model of DC development, and seemed supported by the findings that DC isolated from lymphoid organs expressed high surface levels of MHC II compared to other cells, expressed certain co-stimulator molecules, and activated T-cell proliferation in MLR cultures. This picture has shifted radically, in part reflecting the more recent paradigm that immature DC are required to induce tolerance. The pDC in lymphoid organs are clearly not a mature DC form, and appear closer to a DC precursor. The maturity of cDC varies with the DC subtype, with some populations in an immature state, and others in a mature one, in the steady-state mice.

The three subtypes of cDC found in the spleen, their equivalent cDC subtypes in LN, and the cDC in the thymus, all should be considered immature by the criteria previously used for the antigen processing and presentation capabilities of Langerhans cells or *in vitro*-derived DC [20]. Since in steady-state mice those DC may never become mature by these criteria, quiescent versus activated DC may be preferable terms. The three cDC subtypes of spleen are all capable of phagocytic activity *in vivo* [21] and of uptake and processing of antigens [20, 71]. They are therefore able to sample and present self or foreign antigens reaching the lymphoid organs themselves [40, 72]. Although surface MHC II levels are relatively high, most MHC II is in endosomes within the cell [20, 40, 73]. Activation of these cDC by microbial stimuli, or by simple overnight culture, is required to shut-down subsequent antigen processing and presentation, augment the amount of MHC II on the cell surface, and produce the high surface levels of co-stimulator molecules characteristic of "mature" DC [18–20, 74]. Even the cDC within the thymus, some of which have slightly higher levels of surface MHC II and co-stimulator molecules than their peripheral counterparts, behave as "immature" DC by these criteria [20].

The only cDC within steady-state mouse lymphoid organs which may be classed as "mature" are the migratory DC subtypes which, like Langerhans cells, have arrived in LN via lymph [20]. These have largely shut down processing and presentation of newly encountered antigens [20, 63] due chiefly to downregulation of MHC II synthesis [40], but have high surface levels of MHC II and co-stimulator molecules. Therefore they present those antigens previously collected in the peripheral tissues of origin more efficiently than those encountered within the LN itself [63]. However, for the migratory DC present in the LN of steady-state mice, this more mature or activated state does not necessarily imply a capacity to induce immunity; their main role may still be maintenance of tolerance [75–77].

12.10 Generation and Lifespan of DC Subtypes

The rate of turnover of DC themselves, as well as the rate of turnover of MHC molecules on the DC surface, is one of the determinants of how long an antigen, once taken up by a DC, will be presented to T cells. This turnover rate varies widely amongst the DC subtypes. It is most readily measured by the rate of accumulation of labeled DC during continuous administration to mice of the DNA precursor BrdU, which can be detected in cells during analysis by a fluorescent antibody. This procedure also provides information on the developmental relationships between the DC subtypes downstream from the last dividing precursor. Two limitations of this approach should be noted. Firstly, since the DNA precursor is administered to the whole animal, the last dividing DC precursor which generated the labeled DC may have been located in a different site from the DC under study. Secondly, although turnover, as measured by the substitution of labeled for unlabeled DC, usually reflects the death of unlabeled DC, it could also be the result of migration of DC out of the organ under study, or the development of the DC into a form no longer recognizable as a DC phenotype.

In steady state spleen cDC show a very rapid turnover, most being replaced by newly generated cells within four days [21]. In contrast, the turnover of pDC is much slower, replacement taking around two weeks [29]. Amongst the spleen cDC, the $CD8^+$ subtype shows the fastest turnover, virtually all cells being replenished within three days. Within the spleen all DC subtypes immediately begin accumulating labeled cells after BrdU administration, with no signs of a lag or of precursor–product relationships between them [21, 44]. None of these spleen DC subtypes behaved as the direct, nondividing precursor of the other, an argument that they represent separate sub-lineages. However this leaves open the possibility of a common precursor at an earlier, dividing cell stage.

The turnover of cDC in other lymphoid tissues is slower than in spleen. In particular the lifespan of the cDC found in skin draining LN appears much longer, although again the $CD8^+$ subtype shows fastest turnover [44, 53]. The lifespan of the Langerhans cells found in LN seems especially long, with half of these migratory cells still being unlabeled after three weeks of BrdU administration. However the BrdU labeling kinetics of the potentially migratory Langerhans cells still within the skin at the time of sampling the LN DC also shows a very slow turnover, indicating that most of the long life of this DC subtype is spent within the skin, not the LN [44]. The actual residence time of Langerhans cells in the LN after migration appears very short.

Very short (3 h) exposure to BrdU does not label most cDC, indicating the majority are not themselves cycling cells. This supports the concept that they are end cells, with no further generative potential, despite being “immature” and capable of further differentiation. However this extreme view can be questioned, since around 4% of these cDC were labeled with a short BrdU pulse [21, 44]. This might simply represent a distinct small generative compartment, or perhaps earlier precursor cells that could not be properly separated during analysis. But an alternative

interpretation is that all cDC have the capacity to occasionally divide, allowing a form of homeostatic proliferation akin to that known for lymphocytes. The question of whether mature DC are end cells has recently been raised for DC produced in culture [78, 79] and must now be considered for the *in vivo* DC subtypes as well.

12.11 Human DC Subtypes

This chapter has concentrated on the DC subtypes in steady-state mice, the accessible model where the most detailed analysis has been performed. A major issue is whether there exist equivalent DC populations in humans. The main problem is that equivalent DC sources have seldom been compared. Most work on human DC has been performed either on culture-generated DC, or on blood DC, for reasons of availability. In contrast, the complexity of the mouse DC network has been revealed mainly by studies on spleen or LN DC. Direct comparison of human and mouse DC from the same source is needed. A second problem is that there are differences between mouse and human DC in expression of the commonly used DC surface markers. Some of these differences may have limited functional significance and a wider range of more relevant markers needs to be introduced. Thus CD123, the IL-3 receptor, is sufficiently high on human pDC to serve as a surface marker; it is present on mouse pDC, but the levels are not high enough for a good fluorescent stain. CD8α, a key marker for mouse cDC subpopulations, is not expressed on human DC; however human DC may be similar in this respect to DC from CD8α deficient mice, where the DC subtype normally marked by CD8α is still present and functional, and readily detected by other markers [14]. However there are clear differences in expression of some surface molecules directly relevant to DC function. Thus TLR9 is selectively expressed by human pDC whereas it has a wide distribution amongst mouse DC [80].

The distinction between human pDC and cDC (the latter often termed myeloid DC) was established well before murine pDC were delineated. Human pDC are generally similar to mouse pDC in such crucial features as the capacity to produce type I interferons. Human Langerhans cells are distinguished from other DC by features similar to those in mouse, including the presence of Birbeck granules. However it is not yet clear whether segregation of human lymphoid tissue DC into functionally distinct equivalents of the murine $CD8\alpha^+$ and $CD8\alpha^-$ subsets is possible, and this is a key question[81–83].

Although there will undoubtedly be some important differences between mouse and human DC, it is encouraging that when the same DC source has been directly compared in the same laboratory, there were strong similarities between the species. Direct comparison of early DC in mouse blood with human blood revealed the presence of similar pDC and early cDC populations [37], although the finer division into multiple cDC subsets was not attempted. A study on human thymus DC revealed three subtypes closely similar to those found in the mouse, with the presence of pDC, of a minor subgroup of more “myeloid” $CD11b^+$ cDC, and a major

group of $CD11b^-$ cDC which resembled the major $CD8\alpha^+$ $CD11b^-$ cDC mouse thymic subtype. We await a similar direct comparison of mouse and human spleen and LN DC.

12.12 Functional Differences between DC Subtypes

The issues of antigen processing by DC, activation of DC by microbial stimuli, of cytokine production by DC and of activation and direction of T-cell responses by DC are all considered elsewhere in this volume. The different DC subtypes show important differences in these functions, although there is considerable overlap and much flexibility in response to different stimuli. Table 12.1 documents some of these function related differences for the DC subtypes found in mouse spleen. The evolutionary advantage of maintaining this complex network of DC subtypes presumably lies in such differences in functional potential.

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IV
Circulating Dendritic Cells and their Precursors

13
pDC: From Plasmacytoid Dendritic Cell Precursors to Professional Type 1 Interferon-producing Cells

Yong-Jun Liu, Holger Kanzler, Yui-Hsi Wang, Yi-Hong Wang, Michel Gilliet, Wei Cao and Tomoki Ito

13.1
Introduction

The identification of B cell-unrelated plasma cells and the revealing of their functions as the plasmacytoid dendritic cell precursors (pDC) and the professional type 1 interferon-producing cells (IPCs) represent the results from three independent areas of research during the last four decades.

13.1.1
A Mysterious Cell Type with Plasmacytoid Morphology

In 1958, pathologists Lennert and Remmele [1] reported a histological observation of cells with plasma cell morphology in the T-cell areas of human reactive lymph nodes. These plasma cells were named "T-associated plasma cells." In 1983, Feller et al. [2] found that the T-associated plasma cells expressed CD4 (OKT4), a marker for helper/inducer T cells, but that they did not express B-cell antigen or immunoglobulin. T-associated plasma cells were renamed "plasmacytoid T cells" and were suggested to be the counterparts of plasma cells of the B-cell system that secrete T-cell lymphokines instead of immunoglobulins. The name "plasmacytoid T cells" was later questioned by Facchetti [3], who found that plasmacytoid T cells did not express T-cell receptor (TCR) component CD3, but that they did express MHC class II and some myeloid antigens. He suggested that the plasmacytoid T cells should be renamed "plasmacytoid monocytes" [3]. O'Doherty, Bhardwaj and Steinman reported the isolation of two subsets of dendritic cells from human blood by

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immunofluorescence cell sorting. The DR^+CD11c^+ subset expresses the myeloid antigen CD11b, CD13, CD33 and CD45RO and has the capacity to undergo spontaneous maturation in culture medium and induce strong allogeneic T-cell proliferation [4]. The DR^+CD11c^- subset does not express the myeloid antigen CD11b, CD13, CD33 and CD45RO, but does express CD45RA. The DR^+CD11c^- subset does not have the capacity to undergo spontaneous maturation in culture medium nor to stimulate allogeneic T-cell proliferation. Upon culture with monocyte-conditioned medium, DC^+CD11c^- cells differentiate into DCs. The authors suggested that while the DR^+CD11c^+ DC subset might represent the migrating interstitial DCs on their way to spleen or lymph nodes, the DR^+CD11c^- DC subset represents blood Langerhans cell precursors migrating to the skin [4].

In 1997, Grouard et al. isolated $CD11c^-CD4^+CD45RA^+$ cells from tonsils and showed for the first time they were the historical plasmacytoid T cells or monocytes identified by pathologists. These cells died rapidly in culture, but in the presence of IL-3 and CD40 ligand (CD40L) they differentiated into cells with mature interdigitating DC morphology. The survival effect of IL-3 on these "plasmacytoid" DC precursors depended on their expression of high amounts of IL-3 receptor alpha chain (IL-3Rα) as demonstrated by Olweus et al. [5]. These cells are identical to the peripheral blood $CD11c^-$ immature DC subset [4]. This study concludes that human

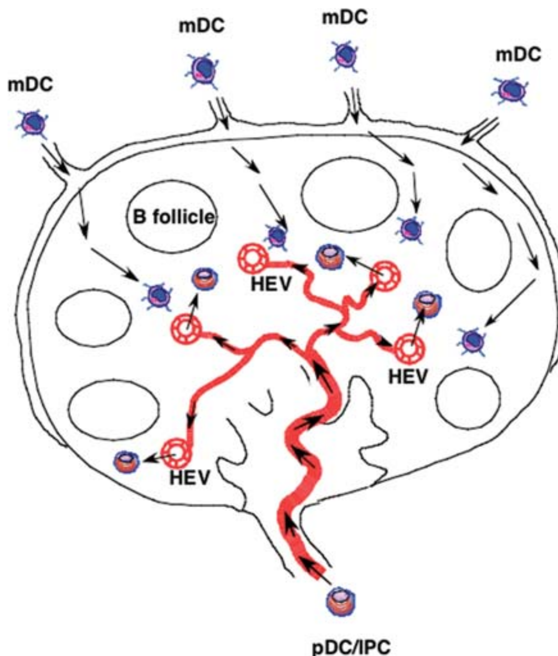

Fig 13.1 Migration pathways of pDC/IPCs versus mDC into a lymph node. pDC/IPC migrate into a lymph node through blood and high endothelial venules (HEV). mDC migrate into a lymph node through afferent lymphatics. Both pDC/IPC and mDC are localized in the T cell rich-areas.

plasmacytoid T cells/monocytes belongs to a unique subset of DC precursors, now known as the plasmacytoid DC precursor (pDC).

13.1.2
A Mysterious Cell Type that has the Capacity to Produce Huge Amounts of Type 1 IFNs

From a totally different direction, virologists and NK-cell biologists had been tracing the same cells for the last 30 years. Although all nucleated cells can produce type 1 interferon (IFN) upon infection by an appropriate virus, it became clear in the late 1970s that there is a blood cell type that has the ability to produce more type 1 IFN than other cell types [3]. Initial studies suggested that natural killer (NK) cells were the major type 1 IPCs following viral infections, and NK cells activated themselves by type 1 IFNs in an autocrine fashion [6, 7]. Subsequent studies using monoclonal antibodies specific for NK cells, B cells, T cells, monocytes, and macrophages concluded, however, that IPCs were not any of these [8, 9]. Interestingly, IPCs were found to express MHC class II [10, 11], suggesting that IPCs might represent blood DCs [11]. One cell-sorting study suggested that IPCs were conventional DCs, which have a HLA-DR$^+$CD4$^+$CD3$^-$CD19$^-$CD16$^-$CD56$^-$CD14$^-$ phenotype, the conventional DC morphology and the ability to induce a strong allogeneic mixed lymphocyte reaction (MLR) [12, 13]. However, an earlier study showed that an IPC-enriched population was unable to induce MLR [14], and it is clear now that blood cell population expressing the HLA-DR$^+$CD4$^+$CD3$^-$CD19$^-$CD16$^-$CD56$^-$CD14$^-$ phenotype contains both CD11c$^+$ conventional DCs and CD11c$^-$ plasmacytoid DC precursors. The field was obviously frustrated by the inability to purify a homogeneous population of IPCs and to perform a definitive study [15]. In 1996, Svensson et al. [16] took a more direct approach by analyzing the surface phenotype of intracellular IFN-α^+ blood mononuclear cells after a 5-hour stimulation with herpes simplex virus (HSV). Svensson et al. [16] reported for the first time the most comprehensive surface phenotype of IPCs, as CD4$^+$DR$^+$CD45RA$^+$CD11c$^-$CD11b$^-$ CD14$^-$CD13$^-$CD33$^-$CD16$^-$CD80$^-$CD86$^-$. The limitation of this study was that the use of HSV to activate the cells, followed by fixation and treatment with detergent for intracellular IFN-α staining, prevents the identification of the phenotype and morphology of the original cells and the further functional study of the cells.

13.1.3
From pDC to IPC

In 1998, we collaborated with Dr. Fred Siegal to determine whether pDCs are actually IPCs, because of their phenotypic similarities [17, 16]. We reported the following findings [18, 19]: (*a*) highly purified pDCs (over 99% purity) produced 100–1000 times more type 1 IFN than the other blood cell types following HSV activation and indeed represent IPCs; (*b*) IPCs rapidly lose the ability to produce large amounts of type 1 IFN following maturation into DCs in culture with IL-3 or HSV. We concluded that pDCs indeed represent IPCs. These findings suggest that, upon

recognition of microbial pathogens, IPCs rapidly produce type 1 IFN as effector cells of the innate immune system and subsequently differentiate into DCs to trigger adaptive immune responses. Human pDCs/IPCs were also reported to be isolated from human peripheral blood on the basis of their expression of immunoglobulin-like transcript receptor 3 (ILT3), but not ILT1 [20]. Paradoxically, the $ILT3^{+}ILT1^{-}$ cells were found to respond to lipopolysaccharide (LPS) by producing large amounts of IL-12p70 [19], which could not be explained by the fact human pDCs/IPCs do not express Toll-like receptor (TLR)-4 and do not respond to LPS [21, 22, 23].

13.1.4
pDCs/IPCs in Mice, Rat, Pig, and Monkey

In 2001, three groups identified and isolated mouse pDCs/IPCs from lymphoid tissues, as $CD11c^{+}B220^{+}Gr\text{-}1^{+}CD45Rb^{high}CD11b^{-}$ cells [24, 25, 26]. Researchers have also recently identified pDCs/IPCs from monkey [27], pig [28, 29], and rat [30].

13.2
Isolation and Characterization of pDCs/IPCs

13.2.1
Isolation of Human pDCs/IPCs

At least three different methods have been developed to isolate pDCs/IPCs from human blood and tonsils. These include (*a*) isolation of $CD4^{+}CD11c^{-}Lin^{-}$ (CD3, CD14, CD16, CD19, CD56) cells by three-color immunofluorescence cell sorting [17]; (*b*) isolation of $CD123^{high}HLA\text{-}DR^{+}Lin^{-}$ cells by three-color immunofluorescence cell sorting [5]; and (*c*) isolation of $BDCA2^{+}$ or $BDCA4^{+}$ cells by immunofluorescence cell sorting or magnetic bead cell sorting [31, 32]. We prefer to isolate pDCs/IPCs by using the first method ($CD4^{+}CD11c^{-}Lin^{-}$ cell sorting) because it gives high purity and does not appear to interfere with the function of pDCs/IPCs. The second method ($CD123^{high}HLA\text{-}DR^{+}Lin^{-}$ cell sorting) may give rise to basophil contamination. Although BDCA2 is very specific for pDCs/IPCs, anti-BDCA2 may inhibit pDC/IPC production of IFN-α. Many laboratories are currently using anti-BDCA4 magnetic bead isolation kits. Because BDCA4 is also expressed on $CD11c^{+}$ myeloid DCs (mDCs) and T cells [33], the third method does not give pure pDCs/IPCs [34].

13.2.2
Isolation of Mouse pDCs/IPCs

Mouse IPCs can be isolated from peripheral lymphoid tissues or bone marrow by flow cytometry according to $CD11c^{low}B220^{+}Gr\text{-}1^{+}$ [24, 25, 26]. Recently, at least four monoclonal antibodies specific for IPCs including 120G8 [35], 440c [36], 2E6/anti-

Ly49Q [37] and mPDCA-1 (available from Miltenyi) have been reported. These monoclonal antibodies will greatly facilitate the enrichment and isolation of mouse pDCs.

13.2.3
pDC/IPC Morphology

On Giemsa staining under light microscopy, human pDCs/IPCs indeed look like plasma cells. They are slightly smaller than $CD14^+$ monocytes, but bigger than resting lymphocytes (Fig. 13.2A). Whereas a monocyte displays a horseshoe-shaped nucleus (Fig. 13.2B), a pDC/IPC displays an eccentric kidney-shaped nucleus (Fig. 13.2B). And whereas monocytes have many vesicles in the cytoplasm,

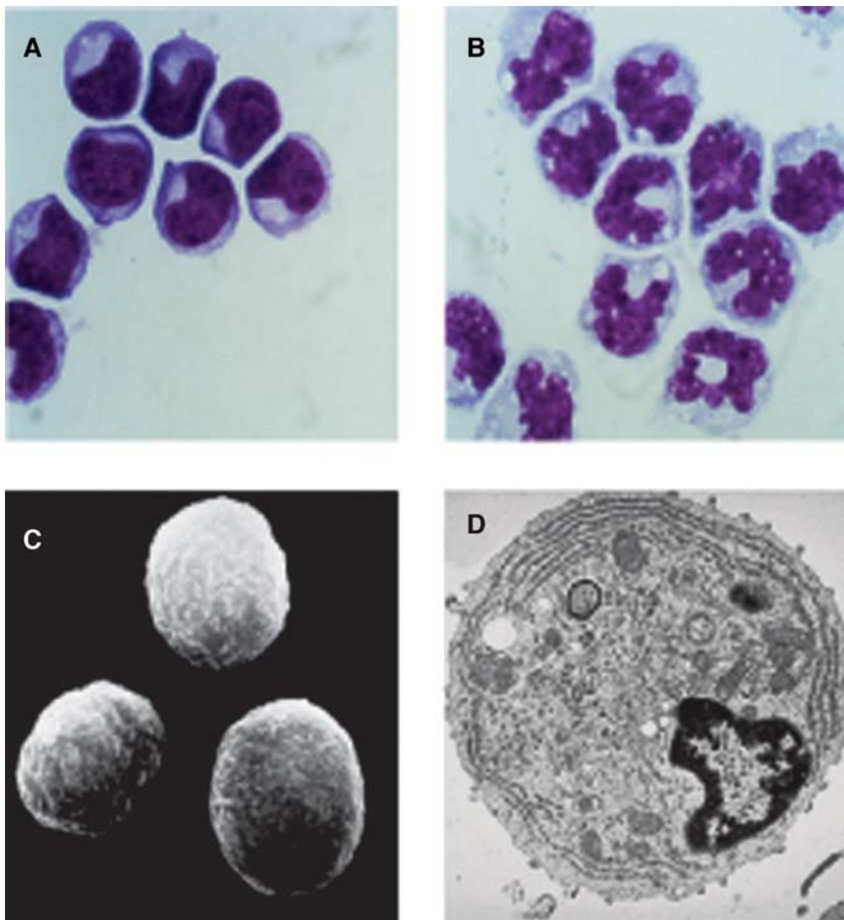

Fig 13.2 Morphology of pDC/IPC. A. Giesma staining of pDC/IPC; B. Giemsa staining of monocytes; C. Scanning EM of pDC/IPC; D. Transmission EM of pDC/IPC.

pDCs/IPCs have a basophilic cytoplasm that contains a pale Golgi zone. By scanning EM, pDCs/IPCs display a smooth, round, lymphoid morphology and are 8–10 µm in diameter (Fig. 13.2C). By transmission EM, pDCs/IPCs display nuclei with marginal heterochromatin and cytoplasm containing well-developed rough endoplasmic reticulum, small Golgi apparatus, and many mitochondria (Fig. 13.2D). Within the isolated mouse pDC/IPC population, only about 20% has plasmacytoid morphology under the EM [25].

13.2.4
Surface Phenotype of pDCs/IPCs

Human pDCs/IPCs do not express the lineage-specific markers for all the known cell types within the immune system, including surface and cytoplasmic immunoglobulin and CD19 (B cells), TCR and CD3 complexes (T cells), CD14 (monocytes), CD16 and CD56 (NK cells), and CD11c (mDCs). Although pDCs/IPCs were named plasmacytoid monocytes because of their expression of MHC class II and myeloid antigen such as CD68, pDCs/IPCs do not express most of the antigens expressed on myeloid cells, such as CD11b, CD13, CD14, or CD33. Nor do they express nonspecific esterase, and they lack phagocytic activity. These facts together suggest that pDCs/IPCs belong to an independent cell lineage within the immune system. A summary of the phenotypic and functional characteristics of human and mouse DC subsets, including pDCs/IPCs, is presented in Tables 13.1 and 13.2.

13.3
pDC/IPC Development

The developmental path and molecular regulation of pDCs/IPCs are not fully understood. Today, FLT3L ligand is the only known cytokine that is critical for pDC/IPC development from hematopoietic stem cells (HSCs) in humans and mice [38–41]. The ability of FLT3L to promote pDC/IPC development *in vivo* was confirmed by experiments showing that administration of FLT3L into human volunteers led to an increase in the number of peripheral blood pDCs/IPCs in humans [42], and that FLT3L-transgenic mice have increased numbers of pDCs/IPCs, whereas FLT3L-deficient mice have less pDCs/IPCs [41, 43].

The notion that pDCs are of lymphoid origin had been supported by findings that the gene transcripts of pre-T-cell receptor α (pTα) λ5, Spi-B, as well as IgH D-J gene rearrangements could be found in pDCs, but not by mDCs [44, 45]. Moreover, three separate studies further support the lymphoid origin of pDCs in human and mice: (*a*) overexpression of the dominant-negative transcription factors Id2 or Id3 in human CD34+ hematopoietic progenitor cells blocks development of pDCs/IPCs, T cells, and B cells, but not of mDCs [46]; (*b*) knock down of Spi-B mRNA in human CD34+ hematopoietic progenitor cells strongly inhibit their potential to differentiate into pDCs [161]; and (*c*) while pDCs and B cells depend on CIITA promotor pIII for their expression of MHC class II, macrophages and all the

Tab. 13.1 Human peripheral blood DC and DC precursors.

Phenotype	*pDC/IPC*	*Monocytes*	*CD11c⁺ imDC*
Myeloid marker			
CD11b	–	+	+
CD11c	–	+	+
CD13	–	+	+
CD14	–	+	–
CD33	–	+	+
Lymphoid marker			
Pre-Ta	+	–	–
Ig1-like 14.1	+	–	–
Spi-B	+	–	–
Pattern recognition receptors			
TLR-1	+	++	+
TLR-2	–	++	+
TLR-3	–	–	++
TLR-4	–	++	+
TLR-5	–	+	+
TLR-6	+	+	+
TLR-7	++	–	–
TLR-8	–	++	++
TLR-9	++	–	–
TLR-10	+	–	+
Mannose R	–	+/–	+/–
BDCA2	+	–	–
CD1a, b, c, d	–	+/–	+/–
Other differentially expressed antigens			
CD4	++	+	+
CD45RA	+	–	–
CD45RO	–	+	+
IL-3R	+++	+	+
GM-CSFR	+	++	++
Function			
IFN-α/β production	++++	+	+
IL-12 production	–	++	++
phagocytosis	–	++	++

other myeloid DCs depend on CIITA promptor pI for their MHC class II expression [47].

However, more recent studies revealed that FLT3$^+$ cells within either common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) could differentiate into both mDCs and pDCs in cultures and *in vivo* [48, 49]. In addition, studies in mice deficient in IFN regulatory factor (IRF)-8, a critical transcriptional factor for the myeloid cell lineage [50], demonstrated that the generation of pDCs/

Tab. 13.2 Phenotype and function of mouse DC subsets.

	CD8a⁺CD4⁻	*CD8a⁻CD4⁻*	*CD8a⁻CD4⁺*	*IPCs*
Phenotype				
CD8	+	–	–	+/–
CD11b	–	+	+	–
CD11c	+	+	+	+
CD4	–	+	+	+/–
B220	–	–	–	+
Ly6c	–	–	–	+
CD45RB	+	+	+	+++
DEC-205	+	+/–	–	–
Function				
IL-12	+++	–	–	+
IFN-γ	++	–	–	–
IFN-α	+	–	–	+++
Cross-priming of CD8 T cells	+	–	–	–

IPCs, CD8α⁺ DCs, epidermal DCs, and dermal DCs were all impaired [51]. As a result of these seemingly divergent findings, several different hypotheses have been proposed regarding the developmental origin of pDCs, including the existence of a common DC precursor in blood that can give rise to all DC subsets [52], pDCs arising as a branch of the committed lymphoid lineage [17, 45], and lineage conversion [53].

A recent study by Akashi's group [54] shows that although CMP does not express RAG gene products and IgHD-J rearrangement, pDCs derived from CMP express RAG gene products and show IgHD-J rearrangement. This study suggests that pDCs represent a unique hematopoietic lineage, whose development may be much more flexible than both conventional lymphoid (B, T, NK) and myeloid (monocyte and granulocytes) cells. Another recent study shows that pDCs from mouse bone marrow can be converted into the conventional mDCs during viral infection, implicating the plasticity of pDC development pathway [55]. Paradoxically, pDCs derived from spleen were unable to undergo this conversion into mDCs during viral infection. This suggest that: 1) pDCs in bone marrow are immature, and still have the capacity for conversion into mDCs; and 2) pDCs isolated from BM according to CD11c+CD11b-B220+ may contain unique precursors for mDCs.

Several studies suggest that pDCs in mice may contain two subsets [37, 56, 57], which make the study on the lineage orgin of pDCs even more complicated. Experiments using RAG1/GFP knock-in mice suggest the presence of two stable subsets of pDCs in bone marrow [57]. The RAG1+ pDC1 subset, but not RAG1– pDC2 subset expresses immunoglobulin DH-JH rearrangements, as well as transcripts for the B lineage related genes Pax5, Igα, Igβ and Bcl11a. Both pDC1 and pDC2 subsets express TdT, Spi-B, TLR9 and ICSBP/IRF-8 transcripts, but lack CD16, G-CSFR, IL-7Ra, CD27, c-kit, DX-5 and CD11b. These data suggest that the two func-

tionally specialized subsets of pDCs arise in bone marrow from progenitors that diverge from B, T, NK lineages at an early stage.

The lineage origin of pDCs will remain an open question for the next few years. This is because several questions remain to be answered, including:

1 Do the progenitor cell or the pDC cell populations described in all the studies represent pure and homogeneous populations?

2 In particular, is there a CMP population that is fully committed to myeloid lineages without any other lymphoid potential or a CLP population that is fully committed to lymphoid lineage without any other myeloid potential?

3 Do CD11c+B220+ bone marrow cells contain only pDCs without containing any early progenitors?

13.4 Localization, Migration, and Lifespan of pDCs/IPCs

The identification of human pDCs/IPCs in fetal liver, thymus, and bone marrow suggests that pDCs/IPCs develop from HSCs within these primary lymphoid tissues [38]. Transfer of $CD34^+$ hematopoietic progenitor cells into SCID mice leads to generation of human pDCs/IPCs in mouse bone marrow and human thymic transplants [58–60]. During adult life, pDCs/IPCs appear to be produced constantly from bone marrow. Injection of FLT3L or granulocyte colony-stimulating factor (G-CSF) into healthy human volunteers leads to a significant increase in the number of peripheral blood pDCs/IPCs [42]. G-CSF may promote pDC/IPC immobilization from bone marrow. After leaving the bone marrow, pDCs/IPCs appear to migrate into the T cell-rich areas of the secondary lymphoid tissues through HEV in lymph nodes (Fig. 13.1) and mucosa-associated lymphoid tissues, as well as through marginal zones of the spleen under steady-state conditions [17, 20, 32, 35, 36, 61, 62]. This migration behavior is much like that of B and T lymphocytes. By contrast, monocytes and immature mDCs appear to migrate into nonlymphoid tissues and then migrate into the T cell-rich areas of secondary lymph nodes through afferent lymph upon activation (Fig. 13.1). This unique migration property of pDCs/IPCs into lymphoid tissues appears to be associated with their expression of CD62L, CXCR4, CCR7 and α4 integrins which interact sequentially with L-selectin ligands expressed by HEV and chemokines ELC/CCL19 and SLC/CCL21 expressed by HEV and stromal cells within the T cell-rich areas [62, 63]. PDCs/IPCs are found in diverse inflamed nonlymphoid tissues such as inflamed nasal mucosal, a variety of cutaneous lesional skins, including herpes zoster, skin blisters simulating syphilis infection, psoriasis vulgaris, lupus erythematosus, contact dermatitis, cerebral fluid from multiple sclerosis or from lyme neuroborreliosis, synovial fluid from patients with spondyloarthropathy or rheumatoid arthritis. PDCs/IPCs are also found in human tumor tissues incuding breast cancer, melanoma and ovarian cancer. An elegant study by Zabel et al. shows that human pDC selectively express the novel chemokine-like receptor 1 (CMKLR1), which directs pDC migra-

tion through CMKR 1 agonist chemerin. This study suggests that during inflammation, pDCs are recruited from blood to inflamed tissues through the interaction between CMKLR 1 and chemerin [64].

In mice, pDCs/IPCs differentiate into a unique subset of $CD8^+$ DCs following viral infection [65, 66]. The study of the kinetics of bromodeoxyuridine labeling and adoptive transfer experiments suggests that pDCs/IPCs in mice have a relatively long average life span of about 2 weeks [65].

13.5
Innate Immune Response by pDCs/IPCs

13.5.1
pDCs/IPCs Selectively Express Intracellular TLR-7 and TLR-9 that Respectively Recognize Single-Stranded RNA and Double-Stranded DNA

The remarkable functional plasticity of DCs at the precursor or immature stage has brought into question the existence of different DC subsets or lineages. Within the immune system, evolutionary force drove the development of multiple subsets of B and T lymphocytes, which rapidly and efficiently respond to common microbial antigens. Unlike conventional B and T cells, B-1 B cells, γδ T cells, and NKT cells express restricted and distinct antigen receptors, capable of recognizing common antigens derived from bacteria or damaged host cells, a phenomenon called by Klaus Rajewsky [67] "evolutionary immunological memory." We hypothesized that if separate DC lineages/subsets with specialized functions really exist, they might express different sets of Toll-like receptors (TLRs), the ancient microbial pattern recognition receptors highly conserved from *Drosophila* to humans [68–71]. We and other investigators demonstrated that, whereas monocytes preferentially expressed TLR-1, -2, -4, -5, -6, and -8, pDCs/IPCs strongly expressed TLR-7 and -9 (Table 13.1) [21–23, 72]. In accordance with these TLR expression profiles, monocytes responded to the known microbial ligands for TLR-2 (peptidoglycan, lipoteichoic acid) and TLR-4 (LPS) by producing tumor necrosis factor (TNF)-α and IL-6. In contrast, pDCs/IPCs produced large amounts of IFN-α in response to DNA virus and CpG oligodeoxynucleotides (CpG ODNs) (TLR-9 ligand) or single-stranded viral RNA (TLR-7 ligand) [21–23, 72–76]. A recent study demonstrated that whereas monocytes and mDCs preferentially express bacteria-recognizing TLR-2, -4, -5, and -6 on the cell surface, pDCs/IPCs preferentially express virus-recognizing TLR-7 and TLR-9 within the intracellular endosomal compartments (Fig. 13.3) [77, 78] . Interestingly, $CD11c^+$ mDCs were found to express TLR-3, suggesting $CD11c^+$ mDCs may play a critical role in recognizing dsRNA viruses. The remarkable differences among monocytes, $CD11c^+$ immatureDC, and pDCs/IPCs in their TLR repertoire expression and responsiveness to microbial antigens suggests that these myeloid-related DC/DC precursors and pDCs/IPCs may have developed through different evolutionary trails to preferentially recognize bacteria and viruses respectively (Fig. 13.3).

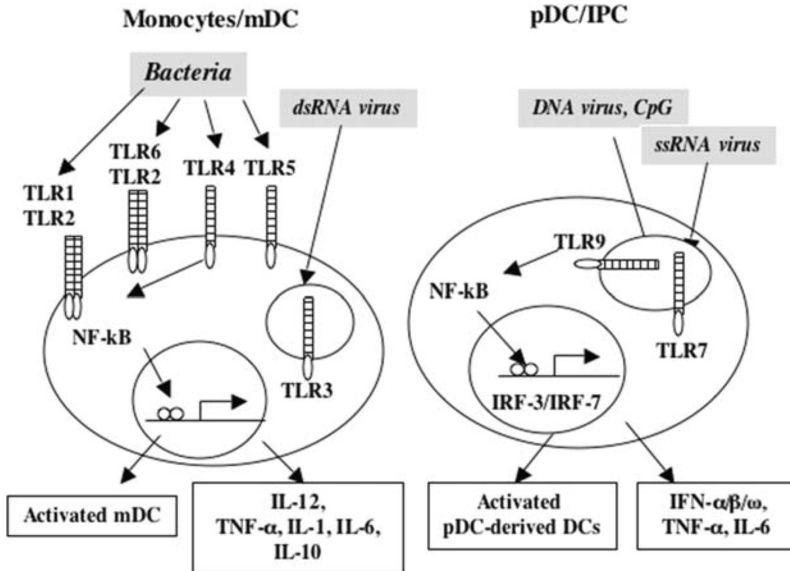

Fig 13.3 pDC/IPC selectively express TLR7 and TLR9 within endosomes that respectively recognize single-stranded RNA- and single-stranded DNA- viruses respectively. Signaling through TLR7 and TLR9 leads to activation of pDCs to secrete large amounts of type 1 IFN and to differentiate into mature DCs. Monocytes preferentially expressed TLR 1, 2, 4, 5 and 6 that recognize different bacterial products. Signaling these TLRs lead to activation of monocytes to secrete TNF-α, IL-1, IL-6 and IL-10, and to differentiate into mature DCs. Similarly to monocytes, mDC express TLR 1, 2, 4, 5 and 6. However, mDC but not monocytes express TLR3 that recognize double-stranded RNA virus. Signaling TLR3 lead to activation of mDCs to produce large amounts of IL-12, IL-1 and IL-6, and a small amount of type 1 IFN.

13.5.2
pDCs/IPCs Are Professional Type 1 IFN-Producing Cells

Upon activation with a virus, pDCs/IPCs produce a huge amount of type 1 IFN (1–2 U/cell, or 3–10 pg/cell) within 24 h, which is 100 to 1000 times more than that produced by any other blood cell type [19]. The type 1 IFN responses from pDCs/IPCs have the following features [34, 79]:

1 pDCs/IPCs do not contain pre-existing mRNA transcripts for type 1 IFNs before viral stimulation.

2 Type 1 IFN mRNA can be detected in pDCs/IPCs as early as 4 h following viral stimulation, and the level reaches its peak at 12 h.

3 From 6 to 12 h following viral stimulation, about 50% of total mRNA expressed by pDCs/IPCs encodes for type 1 IFNs, including all types of IFN-α, IFN-β, IFN-ω, IFN-λ and IFN-τ (H Kanzler, W Cao & Y-J Liu, unpublished observation).

4 pDCs/IPCs produce most IFN-α protein within the first 24 h following viral stimulation.

5 After the first 24 h of viral stimulation, pDCs/IPCs make only moderate amounts of IFN-α, and these activated pDCs/IPCs become refractory to secondary stimulation with the same virus or different virus (H Kanzler, W Cao, and Y-J Liu, unpublished observation).

6 Human pDCs/IPCs produce moderate amounts of TNF-α and IL-6, but not IL-1α, IL-1β, IL-3, IL-10, IL-12, IL-15, IL-18, IFN-γ, lymphotoxin-α, or granulocyte-macrophage colonay stimulating factor (GM-CSF) at protein levels following viral stimulation.

These data suggest that pDCs/IPCs are dedicated to producing type 1 IFNs in antiviral innate immunity. The innate immune system has apparently evolved to have different cell types dedicated to fight against each of the three major microbes: bacteria, viruses, and parasites. Although monocyte/macrophages and neutrophils are dedicated to phagocytosis and killing of bacteria, eosinophils, and basophils, mast cells are dedicated to killing parasites. IPCs may have evolved to control viral infection (Fig. 13.4).

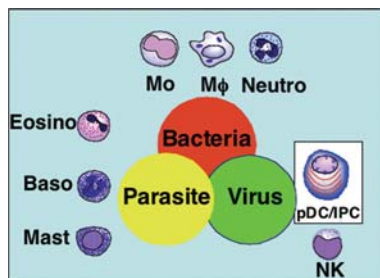

Fig 13.4 The innate immune system has dedicated cell types to control infections by three major microbes.

13.5.3
pDCs/IPCs Rapidly Produce Large Amounts of IFN-α that Is Independent of Positive Feedback of IFN-â Through Type 1 IFN Receptors

The molecular mechanisms underlying the ability of pDCs/IPCs to produce such a large amount of type I IFN rapidly, following viral stimulation, are still poorly understood. The molecular regulation of IFN-α production has been extensively studied in fibroblasts, and a positive feedback model was proposed [80]. Upon viral infection, transcriptional factor IRF-3 is phosphorylated and translocated into the nucleus, where it activates IFN-β gene transcription. IFN-β secreted by the virus-infected cells activates Jak/Stat signaling pathways, leading to formation of both ISGF3 transcriptional complexes, which in turn activates the transcription of IRF-7. The *de novo* synthesis of IRF-7 strongly activates the transcription of IFN-α and

IFN-β genes. The rapid and robust transcription of both IFN-α and IFN-β genes and production of IFN-α and IFN-β proteins by pDCs/IPCs following viral stimulation suggests that the regulation of IFN-α production in pDCs/IPCs may not be mediated by the positive feedback effect of IFN-β. This hypothesis is supported by the finding that, unlike fibroblasts, pDCs/IPCs express high IRF-7 constitutively, which may contribute partly to the extraordinary ability of pDCs/IPCs to produce a huge amount of IFN-α rapidly, without the autocrine IFN-β feedback mechanism [81–84]. This hypothesis was directly tested in a study that used type 1 IFN receptor-deficient mice; fibroblasts derived from these mice produced much less IFN-α than fibroblasts derived from the wild-type mice. However, pDCs/IPCs derived from both type 1 IFN receptor-deficient mice and wild-type mice produced similar amounts of IFN-α in response to vesicular stomatitis virus, suggesting that the ability of pDCs/IPCs to produce large amounts of IFN-α is independent of the positive feedback regulation through the type 1 IFN receptors [85].

13.5.4 TLR7/TLR9-mediated IFN-α production by pDCs/IPCs depends on Myd88-IRAK4-TRAF6-IRF-7 complexes

One of the most remarkable features of pDCs/IPCs is their constitutive expression of high levels of IRF-7, a possible molecular component underlying the ability to rapidly produce huge amounts of type-1 IFN upon signaling TLR-7/TLR-9 upon viral infection. Because signaling through LTLR7/TLR9 depends on MyD88, the question was how this signaling pathway was linked to the activation of IRF7. Two recent studies demonstrated that upon TLR9 signaling, MyD88 recruits and directly interacts with IRF7 through the adaptor molecule TRAF6. This molecule supercomplexes with IRAK4 and appears to represent a cytoplasmic transductional-transcriptional processor for type 1 IFN-production by pDCs/IPCs [86, 87].

13.5.5 Human pDCs/IPCs Have a Limited Ability to Produce IL-12

There have been considerable debates on whether pDCs/IPCs can produce large amounts of IL12, like myeloid DCs. Our previous studies showed that pDCs/IPCs (20 000 cells), freshly isolated on the basis of the $CD4^{+}CD11c^{-}Lin^{-}$ phenotype, produced low to undetectable amounts (less than 10 pg ml^{-1}) of IL-12 p70 following activation with HSV or CpG ODN AAC-30 [21]. By contrast, Cella et al. [20] showed that pDCs/IPCs (10 000 cells) isolated on the basis of the $ILT3^{+}ILT1^{-}$ phenotype produced high levels of IL-12 p70 in response to LPS (808 pg ml^{-1}) and to CD40L (2100 pg ml^{-1}). Krug et al. [22] showed that pDCs/IPCs (100 000 cells) isolated by magnetic beads on the basis of BDCA4 expression did not produce significant amounts of IL-12 p70 in response to LPS alone, CpG ODN alone, or CD40L alone, but produced high levels of IL-12 p70 (more than 6000 pg ml^{-1}) in response to a combination of CD40L and B-type CpG ODN-2006. These two observations challenge the concept that specialized cells are the main producers of either type 1

IFNs or IL-12. The following evidence suggests that the observations that human pDCs/IPCs produced large amounts of IL-12 p70 following activation [21, 22] were due mainly to myeloid DCs in their experiments.

1 The conclusion that human pDCs/IPCs isolated on the basis of $ILT3^+ILT1^-$ produce large amounts of IL-12 p70 (808 pg ml^{-1}) in response to LPS is incorrect [20] because the pDCs/IPCs did not express LPS receptor TLR-4 [21–23, 72].

2 The finding that human pDCs/IPCs isolated on the basis of $ILT3^+ILT1^-$ produce large amounts of IL-12 p70 (2100 pg ml^{-1}) in response to CD40L alone [20] was not confirmed by other studies [21, 22, 34, 88, 89].

3 pDCs/IPCs isolated by positive magnetic bead selection of BDCA4-expressing cells may contain myeloid DCs [22]. First, it is very difficult to achieve high purity of pDCs/IPCs (over 99%) by magnetic bead selection. Second, BDCA4, also known as neuropilin-1, is expressed by monocyte-derived DCs, activated $CD11c^+$ mDCs, and a subpopulation of $CD3^+CD57^+$ T cells [33]. Therefore, human pDCs/IPCs isolated by magnetic bead selection of $BDCA4^+$ cells may contain myeloid DCs.

4 We have recently isolated $BDCA4^+$ cells according to the methods described by Krug et al. [22]. These cells were activated by CD40L plus A-type CpG ODN-2216, B-type CpG ODN-2006, or C-type CpG ODN-C274. At 6, 8, and 10 h after stimulation, intracellular expression of IFN-α and IL-12 p70 by activated BDCA4 cells was analyzed by three-color immunofluorescence flow cytometry. We found that cells producing IL-12 p70 were mainly the contaminating $CD11c^+$ myeloid DCs, but not $CD11c^-$ pDCs/IPCs. No cells produced both IFN-α and IL-12 p70, thus confirming the result of a recent study by Duramad et al. [90].

A more direct demonstration that myeloid DCs and pDCs/IPCs have specialized functions in producing IL-12 versus type 1 IFN, respectively, came from a study using a molecule called R848, which triggers TLR-8 on mDCs and TLR-7 on pDCs/IPCs. This study showed that, whereas myeloid DCs preferentially produced IL-12, in response to R848, pDCs/IPCs produced IFN-α _[91]. This study further supports the concept that pDCs/IPCs are specialized to produce IFN-α, but not IL-12, in response to microbial stimulation.

Findings of recent studies suggest that, unlike human pDCs/IPCs, mouse pDCs/IPCs have the capacity to produce both IFN-α and IL-12 [24–26]. This may indeed represent the species difference between human and mouse pDC/IPC. Mouse and human pDCs/IPCs also have some significant differences in surface phenotype, in particular the expression of TLRs [91, 92]. However, an elegant study by Krug et al. demonstrated that depletion of mouse pDC/IPC *in vivo* leads to a drastic reduction of IFN-α production, but an increase in IL-12 production in response to MCMV infection, suggesting that human pDCs/IPCs and mouse pDCs/IPCs function more similar as the professional type 1 IFN-producing cells that mouse *in vitro* experiments suggested [93].

13.5.6
Myeloid DCs Are Specialized in Producing IL-12, but not Type 1 IFNs

All nucleated cells, including myeloid DCs, have the capacity to produce type 1 IFNs when stimulated or infected by an appropriate virus through a universal PKR-mediated pathway. The uniqueness of pDCs/IPCs is that they are able to produce more type 1 IFN than any other cell types and to dedicate 50% of their transcription to making type 1 IFN mRNAs following viral infection [19, 34]. The concept that there is a specialized cell type that is dedicated to producing type 1 IFNs during viral infection was recently questioned by a study in mice showing that CD11c^{high} myeloid DCs produced as much IFN-α as pDCs/IPCs when high doses of naked synthetic poly I:C were electroporated into the cells [94]. We have re-examined the production of IFN-α by human pDCs/IPCs, CD11c^{+} mDCs, and monocyte-derived DCs isolated from the same donor in response to seven different TLR ligands alone, or in combination with CD40L, and four different viruses (HSV, influenza virus, Saidai virus, HIV) [162]. We found that the maximal IFN-α production by pDCs/IPCs is at least 70 times more than the maximal IFN-α produced by mDCs in response to any of the stimuli described above. By contrast, the maximal amount of IL-12 p70 produced by mDCs is at least 10 times more that the maximal amounts of IL-12 p70 produced by pDCs/IPCs in response to any of these stimuli [34]. These data further support the concept that pDCs/IPCs are professional IPCs and are evolved to become the key effector cells in antiviral innate immunity. Obviously, IPC/pDCs may not be responsible for the innate immune responses to all types of viruses because IPC/pDCs do not express TLR-3 to recognize dsRNA viruses, and some viruses may develop the immunoinvasion mechanisms.

13.6
Regulation of T-cell-mediated Immune Responses by pDCs/IPCs

13.6.1
pDC/IPC Differentiation to Mature DCs through Two Pathways

pDCs/IPCs express low levels of MHC class II and low to undetectable levels of CD80 and CD86, and pDCs/IPCs are incapable of stimulating significant antigen-specific T-cell proliferation. *In vitro* studies suggest that pDsC/IPCs have two pathways to differentiate into mature DCs and acquire the capacity to directly talk to T cells: (*a*) the IL-3-dependent pathway, in which human pDCs/IPCs express strikingly high levels of IL-3 receptors [5, 88] and differentiate into DCs in culture with IL-3 or IL-3 plus CD40L [5, 17]; and (*b*) the IFN-α- and TNF-α-dependent pathway, in which pDCs/IPCs express TLR-7 and TLR-9. Signaling through TLR-7 and TLR-9 by viruses or by synthetic CpG ODN stimulates pDCs/IPCs to produce IFN-α and TNF-α, two cytokines that induce pDCs/IPCs to differentiate into DCs [79].

Whereas pDC-derived mature DCs induced by IL-3 and CD40L preferentially prime naïve CD4+ T cells to produce IL-4, IL-5, and IL-10 [88], pDC-derived mature DCs induced by virus preferentially prime naïve CD4+ T cells to produce IFN-γ and IL-10 [79]. These studies suggest that, like immature myeloid DCs, pDCs also display functional plasticity in terms of priming different effector T-cell responses, depending on the type of maturation signals (Fig. 13.5A). The biological significance of pDC/IPC differentiation into DCs in the presence of IL-3 is unknown. Investigators [79] have proposed that IL-3 may be produced by basophils, eosinophils, and mast cells during parasite infection, and pDC/IPC-derived DCs may play a role triggering antiparasite adaptive T helper 2 (Th2) immune responses. Two recent studies in mice [65, 66] demonstrate that pDCs/IPCs indeed differentiate into DCs following viral infection *in vivo*. These studies indicate that IPCs represent a unique cell lineage within the immune system, which first plays a critical role as effector cells in antimicrobial innate immune responses and subsequently differentiates into professional antigen-presenting cells to initiate adaptive immune responses [94] (Fig. 13.5A).

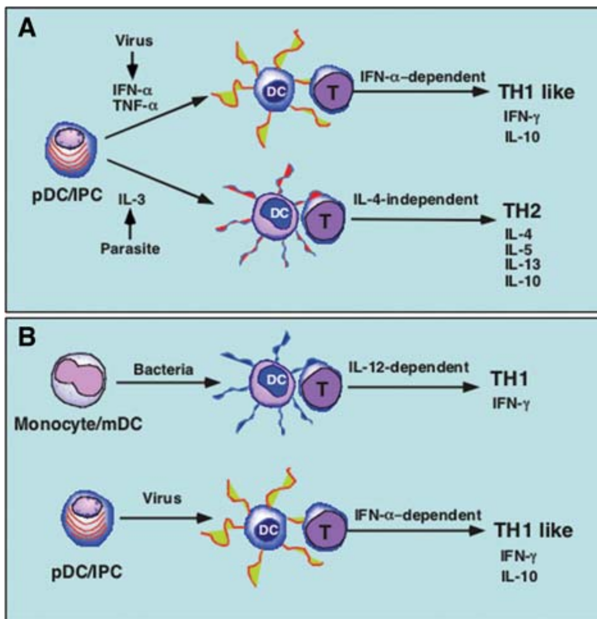

Fig 13.5 A. Functional plasticity of pDC/IPC-derived DCs. Upon viral infection, pDC differentiate into DCs mediated by autocrine IFN-α and TNF-α, which prime naïve CD4+ T cells to produce IFN-γ and IL-10. Upon parasite infection, pDC differentiate into DCs mediated by paracrine IL-3 released by mast cells, eosinophils and basophils, which prime naïve CD4+ T cells to produce TH2 cytokines IL-4, 5, 10 and 13. B. There are two distinct antigen-presenting cell systems in humans that have the capacity to prime naïve CD4+ T cells to produce IFN-γ, while bacteria-activated mDC mainly use IL-12, viral induced pDCs-derived DC use type 1 IFN.

13.6.2 pDC-Derived DCs Induce Th1 by IFN-α but not IL-12

pDCs/IPCs rapidly produce huge amounts of type 1 IFNs (50000 to 100000 pg ml^{-1}) within the first 24 h after viral stimulation. Within the following 48 to 72 h, activated pDCs/IPCs undergo differentiation into mature DCs, which express high levels of MHC class I and class II and costimulatory molecules CD80 and CD86, and pDC-derived mature DCs produce lower but significant amounts of type 1 IFNs (1000 to 5000 pg ml^{-1}). The virus-induced pDC-derived DCs induce human naïve $CD4^+$ allogeneic T cells to undergo strong proliferation and differentiation into IFN-γ- and IL-10-producing cells [79]. Interestingly, the ability of virus-induced pDC-derived DCs to induce naïve $CD4^+$ T cells to produce IFN-γ is dependent on type 1 IFN, but independent of IL-12 [79]. The results of this study suggests that there are two distinct antigen-presenting cell systems in humans that have the capacity to prime naïve $CD4^+$ T cells to produce IFN-γ : bacteria-activated myeloid DCs mainly use IL-12, whereas viral-induced pDC-derived DCs use type 1 IFN (Fig. 13.5B).

13.6.3 pDC-Derived DCs Induce Th2 through OX40L

Previous studies showed that human pDC-derived DCs induced by IL-3 preferentially prime Th2 responses [79, 88, 95]. A recent study demonstrated that pDC-derived DCs induced by IL-3 express high levels of surface OX40L, and neutralizing monoclonal antibody to OX40L significantly inhibited the ability of pDC-derived DCs to prime naïve $CD4^+$ T cells to produce Th2 cytokines, including IL-4, IL-5, and IL-13 [34].

13.6.4 pDC-Derived DCs and their Ability to Prime Naïve versus Memory T Cells

It is unclear whether pDC-derived DCs can directly prime naïve T cells. Two recent studies in mice suggest that mouse pDC-derived DCs induced by CpG ODN-1826 fail to induce antigen-specific naïve T-cell responses but that they can induce antigen-specific memory recall responses *in vivo* [66, 96]. During influenza viral infection, however, pDC-derived DCs appear able to prime virus-specific primary and secondary $CD4^+$ and $CD8^+$ T-cell immune responses *in vitro* and *in vivo* [66, 97]. CpG ODN-1826 may be less potent than influenza virus in activating pDCs.

13.6.5 pDC-Derived DCs and Presentation of Endogenous and Exogenous Antigens

Unlike mDCs, pDCs are poor in phagocytosis and macropinocytosis [17]. A recent study demonstrated that pDC-derived DCs induced by CpG ODN were capable of priming $CD8^+$ T-cell responses to endogenous antigens or peptides, but not those

to exogenous antigens [98]. This finding, together with the previous findings that pDCs selectively express intracellular TLR-7 and TLR-9 and are poor in phagocytosis and pinocytosis, suggests that pDCs and pDC-derived DCs may be more specialized in presenting viral antigens and endogenous self-antigens.

13.6.6
pDCs/IPCs and Cross-priming

Although pDCs have the capacity to process and present viral antigen after viral infection, their ability to cross-present exogenous antigens appears to be limited. Previous studies demonstrated that the $CD8^+$ DC subset is the major antigen-presenting cell that has the capacity to cross-present exogenous antigens to $CD8^+$ T cells [99, 100]. A recent study showed that the ability of myeloid DCs to cross-prime $CD8^+$ T cells during viral infection depends on type 1 IFN [101]. Because pDCs/IPCs represent the major producer of type 1 IFNs during viral infection, it is highly likely that pDCs can promote the ability of myeloid DCs to cross-prime $CD8^+$ T-cell responses to exogenous antigens.

13.6.7
pDCs/IPCs and Regulatory T Cells

Freshly isolated resting pDCs/IPCs express low levels of MHC class I, class II, and CD86 and no detectable level of CD80. Although resting pDCs/IPCs do not have the capacity to induce strong T-cell proliferation, they appear to prime naïve $CD4^+$ T cells to differentiate into IL-10-producing Tr1 cells in cultures in both humans [102] and mice [103, 104]. In contrast to myeloid DCs, the pDCs/IPCs' ability to prime T cells to produce IL-10 is maintained even after differentiation into mature activated DCs. IL-3- and CD40-activated pDC-derived DCs prime naïve $CD4^+$ T cells to Th2 (producing IL-4, IL-5, and IL-10) [34, 88, 95] and naïve $CD8^+$ T cells to $CD8^+$ T suppressor cells (producing IL-10) [89]. Virus-induced pDC-derived DCs prime naïve T cells to produce IFN-_ and IL-10 [79]. These studies suggest that pDC-derived DCs have an intrinsic ability to prime naïve T cells to produce IL-10, regardless of their maturation stages and activation signals. In addition, CpG-ODN induced pDC-derived DCs were shown to induce the generation of $CD4^+CD25^+$ Tr cells *in vitro* [105]. Results of two recent studies suggest that pDCs/IPCs may indeed play a critical role in suppressing asthmatic immune responses to inhaled antigens [106], or in suppressing immune responses that mediate *Leishmania major* infection in mice [107]. We hypothesize that pDCs/IPCs may represent naturally occurring regulatory DCs when directly presenting antigens to T cells, either at a resting stage or at a mature DC stage. pDCs/IPCs may trigger productive, immune T cell-mediated immune responses through activation of myeloid DCs (see next section).

13.7
pDCs/IPCs Regulate the Function of Conventional Myeloid DC by Type 1 IFN

The possible cross-talk between pDCs and mDCs, and the ability of pDCs/IPCs to induce a productive, adaptive, T cell-mediated immune response through activating myeloid DCs, were suggested in three different types of studies. It was shown that pDCs/IPCs in systemic lupus erythematosus (SLE) patients appear to be constantly activated through TLR-9 by self-chromatin–antichromatin antibody complexes to produce type 1 IFNs [108–110]. Type 1 IFNs within the sera of SLE patients strongly activate monocytes and immature mDCs, which subsequently induce strong Th1-mediated immune responses [111, 112]. A recent study [97] showed that human pDCs/IPCs could induce a bystander maturation of myeloid DCs in response to HIV infection *in vitro* by producing type 1 IFN and TNF-α. During immune responses to double-stranded RNA or viral infection in mice, myeloid DCs fail to undergo maturation in the absence of type 1 IFN receptors [113, 114]. The exposure to type 1 IFN at immature stages of mDCs lead to their activation and enhanced production of IL-12, IL-15, IL-18, and IL-23 [95], [115–119].

Together, the above studies suggest that during a viral infection, pDCs/IPCs are activated to produce large amounts of type 1 IFNs within the first 24 h. These type 1 IFNs not only have immediate antiviral effects but also strongly activate viral-infected monocytes and myeloid DCs to present viral antigens to T cells, which subsequently induce strong T cell-mediated antiviral responses (Fig. 13.6).

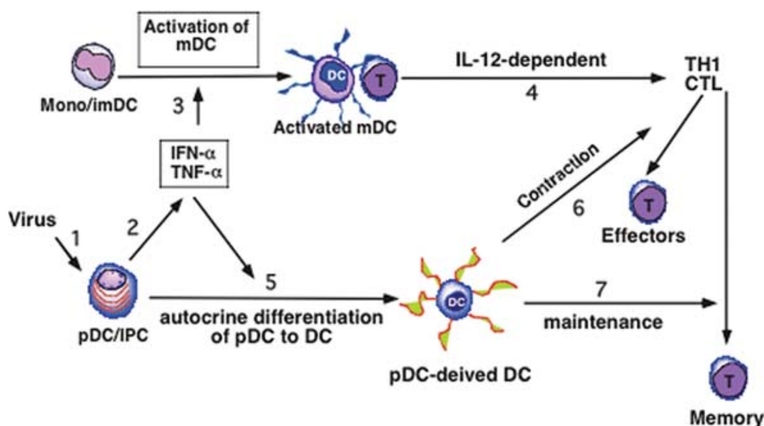

Fig 13.6 pDC/IPC regulate the function of mDC. Viral infection (1) induces pDC/IPC to produce type 1 IFN and TNF-α (2), which activate monocytes or mDC (3) to upregulate co-stimulatory molecules and secrete IL-12; the activated mDC induce strong TH1 and CTL responses (4); pDC/IPC differentiate into mature DC by autocrine IFN-α and TNF-α (5); pDC-derived DCs prime naïve T cells to produce IL-10, which may contribute to the contraction of the effector phase of T-cell responses (6); pDC-derived DCs may also enhance the generation and maintenance of memory T cells through type 1 IFN and other mechanisms (7).

After a productive antiviral adaptive immune response is established, pDCs/IPCs or pDC-derived DCs appear to have several strategies to contract the effector phase and at the same time enhance the memory phase of an immune response (Fig. 13.7): (*a*) at a late stage of an immune response, pDC-derived DCs may prime naïve T cells to produce IL-10 [40, 79, 88]; (*b*) type 1 IFN may directly act on mature myeloid DCs to inhibit their ability to produce IL-12 [119, 120]; (*c*) type 1 IFN may directly prime both myeloid DCs and T cells to produce IL-10 [121–123]; and (*d*) type 1 IFN contributes to the maintenance of memory T cells [124, 125]. The ability of type 1 IFN to inhibit IL-12 production by mature DCs and to prime myeloid DCs and T cells to produce IL-10 may offer a partial explanation for the therapeutic window allowing IFN-β to be an effective treatment for multiple sclerosis.

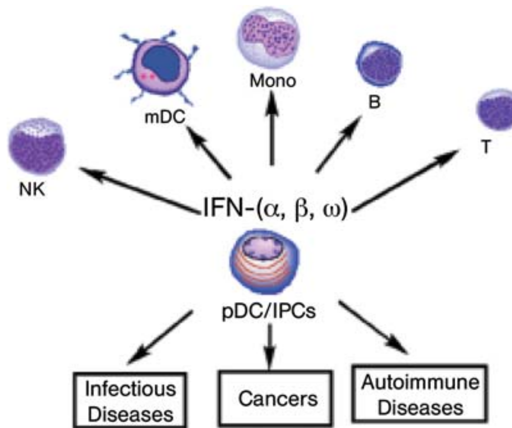

Fig 13.7 The function of pDC/IPCs in regulating the functions of other immune cells and in the control and pathogenesis of human diseases.

13.8
Regulation of NK Cell Function by pDCs/IPCs

Historically, investigators [11, 126, 127] have shown that NK cell activation during viral infection depends on the presence of a population of HLA-DR^{+} accessory cells (now known as pDCs/IPCs). The importance of IPCs in induced NK cell activation was directly demonstrated by using highly purified pDCs/IPCs and NK cells [113]. Previous studies [128, 129] suggested that CpG ODN directly activates NK cells. However, highly purified NK cells are not activated by CpG [130, 131]. This is consistent with the finding that NK cells do not express TLR-9 [39, 131]. In collaboration with Dr. W. Chen, we have recently demonstrated that NK cells isolated by the most commonly used methods all contain 1–2% pDCs/IPCs [39]. When these contaminating pDCs/IPCs are depleted by monoclonal antibody BDCA2 or BDCA4 from NK cell preparation, CpG ODN fails to activate NK cells.

13.9
Regulation of B-cell Function by pDCs/IPCs

Jego et al. [132] recently showed that, when total peripheral blood mononuclear cells from a donor vaccinated with influenza vaccine were cultured with influenza virus, large amounts of virus-specific polyclonal IgG antibodies were induced. This *in vitro* virus-specific antibody response could be completely abolished by depleting pDCs/IPCs from total blood mononuclear cells. This study further showed that two cytokines secreted by virus-activated pDCs/IPCs, IFN-α and IL-6 [79], acted sequentially to drive virus-specific B cells to differentiate into plasma blasts and then mature plasma cells [132].

13.10
pDCs/IPCs and Human Diseases

13.10.1
HIV

The expression of CD4, CXCR4, and CCR5 by human pDCs/IPCs suggests that they may be the targets of HIV infection [17, 63]. Immunohistochemical staining of tonsils and thymus from HIV-infected individuals reveals the presence of HIV p24+ pDCs/IPCs, indicating that pDCs/IPCs are productively infected by HIV virus [133, 134]. Earlier studies showed that peripheral blood mononuclear cells from patients with AIDS had a decreased ability to produce IFN-α in response to HSV stimulation [135, 136]. Recent studies demonstrate that peripheral blood pDC/IPC numbers are decreased in advanced stages of HIV infection [137–140]. Loss of circulating pDCs/IPCs correlated with a high HIV viral load and the occurrence of opportunistic infections and Kaposi sarcoma [137].

13.10.2
Systemic Lupus Erythematosus (SLE)

A link between IFN-α and human SLE was originally made by two clinical observations: (*a*) the development of SLE during IFN-α therapy in a 23-year-old woman with a metastatic carcinoma [141], and (*b*) the finding in many SLE patients of increased serum levels of type 1 IFNs [142–144]. The numbers of circulating pDCs/IPCs are decreased in patients with SLE, but large numbers of activated pDCs/IPCs infiltrate the skin lesions and actively produce type 1 IFN in these patients [110, 145, 146]. pDCs/IPCs appear to be activated by immune complexes consisting of antidouble-stranded DNA antibodies and DNA derived from apoptotic cells [147]. The high levels of IFN-α in the sera of SLE patients were found to activate myeloid DCs to trigger T cell-mediated autoimmunity [111], as well as to promote differentiation of B cells into antibody-secreting plasma cells [132]. Recent studies demonstrate that the expression of type 1 IFN genes and IFN-induced genes repre-

sent the most striking molecular signatures of SLE peripheral blood cells [148–151]. Type 1 IFNs may represent the most important effector molecules in SLE pathology, as well as targets for treating SLE.

13.10.3 Cancer

Both immature myeloid DCs and pDCs/IPCs infiltrate solid tumors [152–154]. Tumor-infiltrating immature myeloid DCs appear to be refractory to stimulation by LPS or CD40L and lack the ability to activate T cells [153, 155]). These tumor-infiltrating immature myeloid DCs probably present tumor antigens continuously and induce tumor-specific regulatory T cells [156, 157]. In breast cancer, high numbers of infiltrating $CD123^+$ pDCs/IPCs are correlated with an increased risk of tumor dissemination and relapse [158]. In ovarian epithelial cell carcinomas, large numbers of pDCs/IPCs were found in ascitic fluids. These pDCs/IPCs were incapable of activating T cells and instead induced IL-10-producing regulatory T cells [159]. A recent study suggests that pDCs/IPCs within the tumor-draining lymph nodes may express indoleamine 2,3-dioxygenase (IDO), which may create a local microenvironment that is potently suppressive of host antitumor T-cell responses [160]. These findings suggest that both immature myeloid DCs and pDCs/IPCs within the solid tumor are incapable of inducing antitumor immune responses, but instead may induce regulatory T cells that inhibit antitumor immunity. In a mouse tumor model, Vicari et al. [153] showed that CpG ODN plus anti-IL-10 neutralizing mAb could activate the tumor infiltrating DCs to induce robust antitumor cytotoxic T-cell responses and tumor rejection *in vivo*. Because pDCs/IPCs are the major cell type expressing TLR-9, this study suggests that CpG ODN may activate tumor infiltrating pDC/IPC to produce type 1 IFN and TNF-α, which then activate tumor infiltrating immature myeloid DCs to induce antitumor T-cell responses. Targeting pDCs/IPCs by CpG ODN to activate the adjacent tumor-infiltrating myeloid DCs may represent a promising strategy for developing cancer vaccines.

13.11 Conclusion

pDCs/IPCs represent a separate hematopoietic lineage, which appears to be closer to B lineage than to myeloid lineage. These cells are continuously produced from HSC within the bone marrow and then released into the peripheral blood stream. Unlike myeloid cells that enter the secondary lymphoid nodes from afferent lymphatics, pDCs/IPCs enter the lymph nodes through HEV (like the T and B lymphocytes) and then colonize the T cell-rich areas. Under steady-state, pDCs/IPCs appear to play a critical role in maintaining peripheral immune tolerance. This may be due to the ability of resting pDCs/IPCs to prime naïve T cells to produce IL-10. Unlike monocytes and mDCs that preferentially express TLR-2, -4, -5, and -6, pDCs/IPCs express TLR-7 and -9 within the endosomal compartment. Upon viral

infection, pDCs/IPCs rapidly produce large amounts of type 1 IFNs, which not only have direct inhibitory effects on viral replication but also contribute to the activation of NK cells, B cells, T cells, and mDCs, leading to the induction and expansion of an antiviral immune response (Fig. 13.7). The ability of activated pDCs/IPCs to activate myeloid immature DC through type 1 IFN appears to be critical for the induction of T cell-mediated antiviral immunity. After producing large amounts of type 1 IFN, pDCs/IPCs rapidly differentiate into mature DCs through an autocrine mechanism mediated by type 1 IFNs and TNF-α. At this stage, pDC/IPC-derived DCs may contribute to the contraction of the effector phase of T-cell responses and at the same time to the establishment of T-cell memory.

Several fascinating questions will challenge us during the next few years, including the following: (*a*) What regulates pDC/IPC development from stem cells, and how and when is pDC/IPC development separated from B, T, NK cell development? (*b*) What regulates the rapid and massive type I IFN production? (*c*) Are there IFN-independent pathways by which pDCs/IPCs regulate the function of other immune cells? (*d*) How can we harness pDC/IPC biology to develop immunotherapy for viral infectious diseases, cancer, and autoimmune diseases?

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14
Monocyte subsets and their relation to DCs

Brigitte Senechal, Darin Fogg, Gaelle Elain, and Frederic Geissmann

Monocytes are circulating leucocytes that link inflammation and the innate defense against microorganisms to the adaptive immune response. They represent 10% of leucocytes in human blood, and 4% of leucocytes in mouse blood.

Monocytes are mature non-dividing cells characterized by an amazing developmental plasticity *in vitro*. They differentiate into dendritic-like and macrophage-like cells in response to a broad range of cytokines and other signals. Although the full range of their functions is not yet known, monocytes thus represent a large pool of effector cells during inflammatory processes, and also a potential reservoir of myeloid precursors for the renewal of tissue macrophages and dendritic cells (DC).

Monocyte differentiation into tissue macrophages and DC *in vivo* has still not been completely characterized, but this question has drawn considerable attention in the past five years, and experimental data are now becoming available to address this problem. Monocytes consist of two main subsets, which can be distinguished by their morphology, surface phenotype, homing potential, and likely by their differentiation potential. There is evidence that monocytes represent direct precursors *in vivo* for inflammation-elicited macrophages (such as those infiltrating inflamed peritoneum) and for DC that differentiate in response to inflammation or infection. In contrast, monocytes appear to be rather inefficient at generating short-lived steady state DC such as spleen CD8α^+ and CD8α^- DC subsets in the mouse. Finally, it remains to be investigated whether monocytes contribute to the renewal of long-lived steady state cells of the peripheral tissues, such as alveolar macrophages, microglia, and Langerhans cells.

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14.1 Monocytes and the Concept of the "Mononuclear Phagocyte System" (MPS)

14.1.1 Blood Monocytes in the Mononuclear Phagocyte System

Monocytes represent 10% of leucocytes in human blood, and 4% of leucocytes in mouse blood. They are distinct from polymorphonuclear (PMNs) and from natural killers (NK) cells, which belong to the innate arm of the immune system, and from lymphoid T and B cells, which represent the adaptive arm of the immune system. Monocytes are present in mammal, birds, amphibians, and fish [1–5] and a related population of hemocytes/macrophages is present in the fly [6–8] which lack lymphocytes. In mammals monocytes represent circulating "accessory cells", which can link inflammation and the innate defense against microorganisms to adaptive immune responses [9]. Indeed, they represent a large pool of potential effector cells during inflammatory and infectious processes, but this pool could also constitute a considerable systemic reservoir of myeloid precursors for the renewal of some tissue macrophages and antigen presenting dendritic cells. In the peripheral blood of mouse and human, monocytes label minimally with ^{3}H-thymidine [10], and do not stain for the proliferation marker Ki67 [11], and thus monocytes appear to be nondividing cells. It has been suggested that a fraction of blood monocytes can be induced to proliferate *in vitro* after exposure to M-CSF and GM-CSF [12] but whether this may happen *in vivo* is unknown. Altogether, the cellular origin and the conditions of renewal of such tissue macrophages and dendritic cells are not well characterized, and it is not yet known to what extent blood monocytes contribute to the turn over of resident cells during post-natal life.

The mononuclear phagocyte system (MPS) was initially defined as a population of cells derived from progenitor cells in the bone marrow, which differentiate to form blood monocytes, circulate in the blood, and then enter tissues to become resident tissue macrophages and antigen presenting cells ([145], reviewed in [13]). However, this original view was vigorously challenged by several important works and has been profoundly revised [13].

The work by the laboratory of Ralph Steinman has established that dendritic cells represent a distinct family of cells that regulate the immune responses [14]. As initially described, they represent steady state or resident cells with a short half-life and renew from bone marrow precursors, such as spleen CD8α^+ and CD8α^- DC subsets in the mouse spleen.

It has been further recognized that dendritic cells, as well as macrophages, have a remarkable heterogeneity related to their origin, phenotype, tissue localization, and function [9, 13, 15, 16]. Several cell types that reside in peripheral tissues such as dendritic Langerhans cells (LC) or brain macrophages (microglia) are long-lived, remain host-derived in their majority after syngeneic bone marrow transplantation, and may selfrenew. Another group of cells represent short-lived cells that differentiate in response to inflammation or infection such as monocyte-derived DC or Tip-DC [11, 17–19]. Macrophages and DC can thus be divided in three main

groups according to their half-life, their replacement after bone marrow graft, and whether their differentiation is elicited or not by inflammation (Fig. 14.1).

Therefore the MPS cannot be considered as a simple family of monocyte-derived cells, but as a more complex cellular system (Fig. 14.1), involved in the scavenging

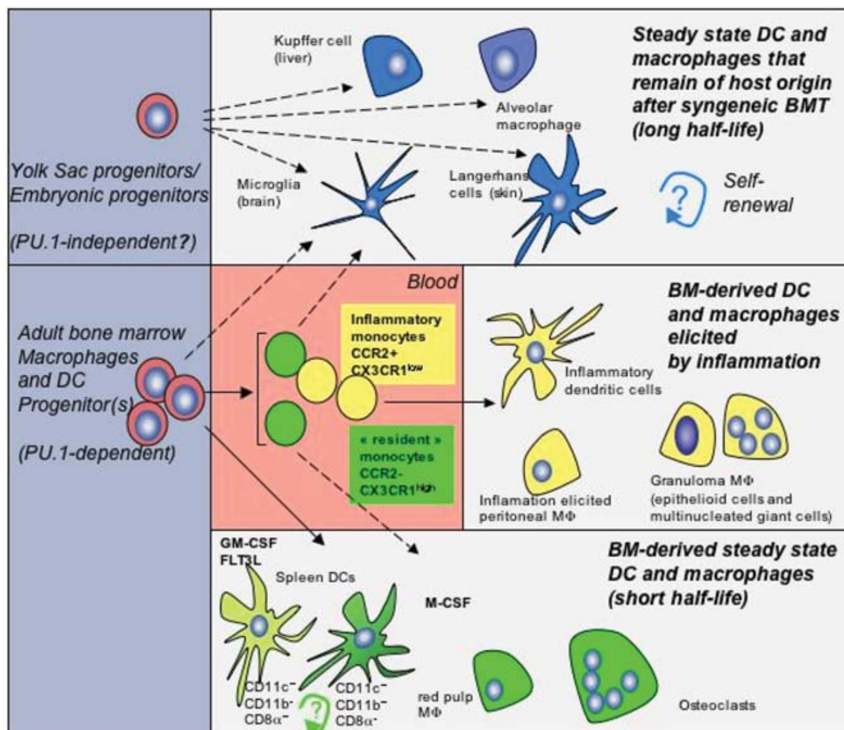

Fig. 14.1 Tentative topology of the mononuclear phagocyte system: Contribution of monocytes to the renewal of tissue macrophages and dendritic cells. Blood monocytes are divided into two main subsets. Tissues macrophages and DC can be divided in three compartments according to their half-life, their reconstitution from donor-derived bone marrow cells after syngeneic bone marrow transplantation, and the role of inflammation in their differentiation. The lineage relationship between precursors and differentiated cell types, and the established and potential contribution of blood monocyte to each of the cell types are indicated by solid and dashed arrows. Steady state macrophages and DC that remain of host origin after syngeneic bone marrow graft (colored in blue) such as LC or microglia, have a long half-life and may self renew in the steady state. Some subsets may originate from yolk sack progenitors. The contribution of bone marrow derived cells, such as monocytes, to their renewal in pathological condition remains to be established. Evidence is presented in the text that CCR2^{+} CX3CR1^{low} monocytes represent direct precursors *in vivo* for at least some populations of bone marrow-derived short-lived macrophages and DC that differentiate in response to infection or inflammation (colored in yellow) and have the potential to migrate to secondary lymphoid organs and to regulate immune responses. Macrophages and DC that differentiate in the steady state from bone marrow derived cells are represented in green. Monocytes appear to be inefficient at generating CD8α^{+} and CD8α^{-} spleen DC subsets.

of dying cells, pathogens, and molecules via a variety of cellular processes, such as phagocytosis and endocytosis e.g. using membrane pattern recognition receptors [20] to which the contribution of monocytes is an area of active research. It is important to keep in mind that potential mechanisms for the renewal of individual subsets which include: (1) self-renewal of resident cells differentiated from embryonic precursors, (2) migration, homing, and limited proliferation of adult bone marrow-derived progenitor cells in peripheral tissues, and (3) continuous extravasation and differentiation of circulating precursors such as blood monocytes [13], are not mutually exclusive, could operate in parallel or sequentially during the life of the animal, and are likely to depend on environmental cues.

14.1.2 Plasticity of Monocytes as Studied *in vitro* and its Relevance to DC Differentiation *in vivo*

In vitro studies of monocyte differentiation have proven that they exhibit considerable plasticity. Early work suggested that cultured monocytes could differentiate into DCs [21] a process that is considerably amplified by the addition of recombinant cytokines [22]. In the past 10 years a number of studies have described the role of cytokines, lipid mediators, or PAMPs (pathogen associated molecular patterns) in this process [23–28]. Although they represented a model of great value for the study of DC functions, which has led to the identification of several basic mechanisms of DC biology, these *in vitro* studies suffer from limitations that should be considered when drawing conclusions about the physiological functions of monocytes. To appreciate the functional significance of these studies, it will be necessary to characterize the contribution of monocyte-derived DC to individual subsets of the MPS system *in vivo*. In addition, since it has been recently recognized that monocytes consist of distinct subsets, which may have distinct differentiation potential [11], they should now be studied individually.

In humans, the majority of studies have investigated the differentiation of monocytes into dendritic cells *in vitro* in responses to cytokines. The combination of GM-CSF and IL-4 was originally described as inducing the differentiation of human monocytes in 5 to 7 days into immature-type dendritic cells [22, 29]. Further studies excluded proliferation of a contaminating progenitor, or the selection of a minor monocytic subset [26, 30], and extended this finding to murine monocytes [31], as well as macaque [32], ovine [33], and porcine monocytes [34, 35].

DC generated by culture with GM-CSF and IL-4, have been termed immature, a differentiation stage characterized by a high endocytosis activity (receptor mediated and fluid phase uptake), and a low expression of membrane MHC class II and co-stimulatory molecule, and a poor ability to trigger allogeneic T cell proliferation [22, 36–38]. Following stimulation with LPS, dsRNA, TNF-alpha, and CD40-Ligand, activated monocyte-derived DCs become mature, a stage of differentiation associated with low endocytosis activity, high expression of membrane MHC class II and co-stimulatory molecules, and robust capacity for inducing T-cell proliferation [39, 40]. Maturation also results in the expression of high levels the chemokine

receptor CCR7 and acquired responsiveness to the CCR7 ligands CCL19 and CCL21, chemokines that are required for homing of activated/mature DC to secondary lymphoid organs. Based on these findings it has been proposed that immature DC may be tolerogenic and mature DC may be immunogenic.

However, DC subsets normally found in mouse tissue display a continuum of maturation features, and even the most immature DCs process and present peptides by MHC class II molecules [41]. Interestingly, immature, or semi-mature LC can apparently upregulate CCR7 and migrate into inflamed lymph nodes in human [42]. Therefore, is not clear whether what is observed *in vitro* in monocyte-derived cells accurately reflects DC behavior *in vivo*, and recent findings have led to the concept that only full DC maturation is immunogenic [43].

14.1.3
Contribution of Monocytes to Long-lived Resident Cells in Peripheral Tissues

It was clear from early studies that, under steady-state conditions, in addition to shortlived macrophages that differentiate from blood monocytes, many other macrophage cell types were long-lived and potentially self-renewing [44]. More recent studies that have attempted to determine the kinetics of murine tissue macrophage and dendritic cell engraftment after bone marrow (BM) transplantation into irradiated syngeneic hosts have confirmed this observation. These experiments indicated that after transplantation, splenic monocytes/macrophages were 90% of donor origin by 1 month, however donor microglial engraftment remained only 30% of donor origin by 1 year, and lung alveolar macrophages and liver Kuppfer cells were approx 50% of donor origin after 1 year, despite heavy irradiation [45]. Of note, donor derived microglia was seen at perivascular and leptomeningeal, but not parenchymal, sites, therefore most of the parenchymal microglia is not replaced by donor-derived cells [45]. In similar experiments, donor-derived langerhans cell engraftment was also extremely low [46].

These data should be considered together with the results of experiments that have shown that macrophages arise from two different lineages, qualified respectively as "primitive" or as "definitive" in the mouse [47, 48]. So called "primitive" macrophages consist of two successive subsets of cells that arise from the yolk sac (YS), have a restricted potential, either monopotent or bipotent (erythro-myeloid) [49], and may play a role in clearance of apoptotic bodies, and may also contribute to the establishment of resident tissue macrophages. It has been shown that early YS macrophages colonize the developing brain and differentiate into primitive microglia, in the zebrafish [1, 50] and chicken [51, 52] models. The contribution of YS macrophages to resident macrophage and DC populations, including microglia, LC, and Kupffer cells, remains to be evaluated. It is interesting to speculate that these cells could arise from YS macrophages and may self renew in the steady state throughout life.

Interestingly, the renewal of these resident populations may be different in the steady state and in pathological conditions. Early experimental work in mouse models, and pathological findings in human patients, have both shown that after

allogeneic bone marrow transplantation, the majority (80% in the mouse) of epidermal LC were of donor origin after 2 months [53–56]. A recent study indicated that while LC self-renew in the steady state, new LC are recruited from bone-marrow-derived precursor after UV irradiation. The recruitment of the precursors for the new LC was dependent on their expression of the CCR2 chemokine [46]. However, the nature of the bone-marrow-derived cells that give rise to the new LC remains enigmatic. Monocytes can differentiate *in vitro* into LC-like cells in the presence of TGFbeta1, GM-CSF, IL4, and TNFα [26, 42, 57]. These reports are consistent with the important role of TGFbeta1 in the differentiation of LC *in vivo* [58]. In addition, it has been proposed that human LC could arise from a dermal precursor that express the monocytic marker CD14 [59]. These dermal CD14 cells also differentiate *in vitro* into LC, when cultured with TGFbeta1, GM-CSF and IL4 [59]. Based on these data, it could be hypothetized that in some inflammatory circumstances such as UV irradiation, human CCR2$^+$ CD14$^+$ monocytes, and their murine equivalent CCR2$^+$ Gr1$^+$ monocytes (see below section 14.3), may contribute to the renewal of LC.

14.1.4
Contribution of Monocytes to Short-lived Bone Marrow-derived Steady State Macrophages and DC

In contrast to long-lived LC and microglia, several macrophages and DC subsets, such as osteoclasts and resident DC of the lymphoid organs, have a short half-life [15, 60]. They reconstitute from donor-derived cells after syngeneic bone marrow transplantation or after adoptive transfer of bone marrow progenitors [61–63]. Of note, self renewal may also contribute to turnover of splenic DCs [60].

Two main hypotheses have been considered regarding the respective origin of these steady state macrophages and dendritic cells. The first is that there are distinct progenitors for each cell type. Most macrophages are dependent on macrophage-colony stimulating factor (MCSF) for their development *in vitro* and *in vivo* while most DC subsets are not [64]. Conversely the ligand for the receptor tyrosine kinase Flk-2 (FLT3-L) is important for the differentiation of many DC subsets but not macrophages [65–67]. Supporting this idea, a progenitor common to human DC and lymphocytes, but not to macrophages, has been described [68]. Among DC themselves, the two subsets of steady state spleen DC in the mouse: CD11c$^+$ CD8α^- and CD8α^+ DC, have been defined as distinct lineages of myeloid-related and lymphoid-related DC, respectively [69]. However, this concept has been challenged by *in vivo* data from mouse models demonstrating that both the common lymphoid progenitor (CLP), and the common myeloid progenitor (CMP), can give rise to both DC types as well as to plasmacytoid DC (PDC), an additional DC subset specialized in the production of type I interferon following TLR ligation [61–63]. The second hypothesis is that these different cell types derive from a common progenitor cell able to develop into DC and macrophages, depending on cytokine signals, other cues encountered at tissue sites, or pathogen exposure. However, a common specific progenitor for DC and macrophages remains to be identified.

Based on their plasticity, monocytes are a candidate common precursor for macrophages and DCs, but several studies, in our laboratory and others, have failed to document efficient blood monocyte differentiation into steady state $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^+$ DC [11, 19]. It appears as if only approximately 1% of spleen $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^+$ DC arise from blood monocytes (our unpublished data). A recent study reported the efficient differentiation of proliferating bone marrow monocytes into $CD8\alpha^-$ and $CD8\alpha^+$ DC *in vivo* [70]. However, it is still possible that the observed DC could have arisen from contaminating precursors within the monocyte preparation.

Finally, a cell type recently identified and named the pre-immunocyte, has been shown to give rise *in vitro* to macrophages and DC when cultured in particular cytokine combinations [71], but it remains unknown whether theses pre-immunocytes contribute to DC and macrophage differentiation *in vivo*.

14.1.5
Evidence that Monocytes Contribute to Short-lived Migrating Dendritic Cells that Differentiate During Inflammation and Infection

Recent experiments have established unambigously that blood monocytes can differentiate into macrophages and dendritic cells *in vivo* in some circumstances [11, 17, 19, 72]. For example, monocyte differentiation into DC during inflammation appears to be efficient, and contributes to both antigen sampling in peripheral tissues and to the transport of antigen or particles to the draining lymph node [11, 17]. This mechanism may also be instrumental in the innate response to intracellular pathogens. Mice lacking the chemokine receptor CCR2 have impaired monocyte migration and are highly susceptible to Listeria monocytogenes [73, 74]. In the absence of CCR2 a population of TNFα and iNOS-producing dendritic cells 'Tip-DC', that express $CD11c^{int}CD11b^{dim}Gr1^{int}$MHC class II molecules, is lacking in the spleen of infected animals, and adoptive transfer of peripheral blood mononuclear cells (PBMC) from wt mice decreases – albeit moderately – the bacterial loads in $CCR2^{-/-}$ mice. This suggests that CCR2 is actually involved in the recruitment of Tip-DC or their precursors to the spleen [18]. Although activation of Tip-DC effector functions (TNFα, iNOs) depends on a functional MyD88-signaling pathway, the recruitment of Tip-DC is independent of this intracellular adaptor molecule [75].

We have shown that a subset of circulating murine monocytes ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^+$, $CCR2^+$, $CX3CR1^{low}$ "inflammatory" monocytes, see section 14.3) with a similar phenotype to that of Tip-DC, are short-lived in the absence of inflammation and are actively recruited to inflamed tissues, where they may differentiate into macrophages, as well as into DCs that upregulate CD11c and MHC class II antigens, and can initiate an antigen specific immune response [11]. The control of acute infection by the intracellular parasite *Toxoplasma gondii* was also recently proposed to be dependent upon the recruitment of the same $Gr\text{-}1^+$ $CCR2^+$ $CX3CR1^{low}$ "inflammatory" monocytes [76]. Therefore it is believed that these inflammatory monocytes are the precursors for the TipDCs [9, 18, 76]. However

there is no formal proof that inflammatory monocytes are the precursors for the TipDCs, or are required for protection against *Listeria* monocytogenes or others pathogens.

14.1.6
Summary of the Respective Contribution of Monocytes to Individual Subsets of the MPS System

To date there is evidence that monocytes represent direct precursors *in vivo* for at least some populations of DC as well as macrophages described in Figure 14.1. Monocytes can give rise to short-lived cells that differentiate in response to inflammation or infection. These cells are most likely to play a key role in the innate part of the primary response, and can also migrate to secondary lymphoid organs and contribute to the adaptative immune response. In contrast, monocytes appear to be rather inefficient at generating steady state bone marrow-derived dendritic cells such as spleen CD8α^+ and CD8α^- DC subsets in the mouse spleen. Finally, it remains to be investigated whether monocytes contribute to the renewal of cells of the peripheral tissues such as microglia and LC which are likely to self renew in the steady state, but can be replaced by bone-marrow derived cells under defined inflammatory conditions.

14.2
Molecular Determinants of Monocyte Differentiation

In mammals, the current model proposes that monocytes differentiate from hematopoietic stem cells through a common Granulocyte Macrophage Progenitor (GMP) intermediate [77, 78]. The growth factor M-CSF is critical for monocyte differentiation from bone marrow progenitors (reviewed in [13]). In mice deficient for either M-CSF, such as op/op mice and M-CSF KO mice, or its receptor c-fms (CD115), the number of blood monocytes is dramatically reduced, although they are still detectable [79–83]. An important function of M-CSF is to maintain survival of cells of the monocyte lineage, as shown by Lagasse and Weissman, because a Bcl2 transgene rescued the differentiation of monocytes and the *op/op* phenotype [84]. The M-CSF receptor, the c-fms gene product, is a tyrosine kinase transmembrane receptor that belongs to the same family as cKit (CD117, the receptor for stem cell factor) and FLT3$^-$ [85, 86]. CD115 is expressed by most adult type, and also by primitive macrophages [13]. Osteoclasts, peritoneal and pleural cavity phagocytes, synovial type A macrophages, testis and bladder macrophages, lung alveolar macrophages, splenic marginal zone metallophils, and lymph node subcapsular sinus macrophages are considered to be absent on *op/op* mice [64, 87, 88]. Splenic red pulp, lymph node medulla, intestinal lamina propria, liver (Kupffer cells), kidney, muscle, tendon, thymus, small and large intestine, and brain (microglia) macrophages are decreased in numbers [64, 87, 88]. Expression of a M-CSF transgene is able to rescue the development of macrophages in these mice [88].

Although they express CD115, LC and spleen, lymph node and Peyer's patch lymph DC subsets are not significantly altered in *op/op* mice [64, 87, 88]. The *op/op* mice are therefore deficient in many tissue-macrophage populations, which have been named M-CSF-dependent macrophages, while other subsets are only decreased in number, or remains apparently unaffected, possibly due to the redundant activities of other cytokines such as GM-CSF and FLT3-L [64, 81, 87, 88].

The growth factor FLT3-L and its receptor (flk2, closely related to c-fms [89]) may also play an important role in monocyte differentiation since the number of monocyte is increased in the blood of FLT3-L treated mice [90] while in contrast CFU-GM precursors are reduced in the bone marrow of FLT3-L deficient mice [65].

Dissection of the human and mouse c-fms proximal promoters has revealed that c-ets-1, c-ets-2, and PU.1 trans-activate the c-fms proximal promoter [91]. The transcription factor PU.1 indeed plays a major role in the differentiation of monocytes. In PU.1 deficient animals, blood monocytes are almost completely absent [83], as well as many macrophages populations such as osteoclasts, thymic dendritic cells and myeloid DC [82, 92–94]. However, in contrast to M-CSF, PU.1 is not expressed, and is not required, by yolk sac "primitive" macrophages, although it is required for normal adult myelopoiesis in the mouse [95]. In keeping with the absence of apparent overlap between PU.1 and *c-fms* expression in embryo there is no effect of the PU.1 null mutation on the number or location of *c-fms*–positive cells anywhere in the embryo at day 10 post-coitus [95].

The role of the transcription factors MafB and C/EBP$^{-}\alpha/\beta$ may also be important for monocyte differentiation [3, 96]. Enforced expression of transcription factors C/EBPα and C/EBPβ in differentiated B cells can lead to rapid and efficient reprogramming into macrophages [96]. C/EBP appears to initiate the reprogramming process by simultaneously downregulating late lymphoid markers (e.g. CD19) via inhibition of PAX5 expression, and upregulating myeloid markers (e.g. CD11b Mac-1), through a synergistic interaction with PU.1 [96]. A dominant-negative allele of MafB inhibits both myeloid colony formation and the differentiation of myeloblasts into macrophages [3]. *In vitro*, PU.1 and MafB are important for the differentiation of monocytes into DC-like cells and macrophage-like cells respectively [97].

14.3 Heterogeneity of Monocytic Cells

14.3.1 Circulating Dendritic Cells

It has long been recognized that monocytic cells are heterogeneous with regards to their morphology and phenotype [98] [34, 99]. In the past years, two minor populations of monocytic cells have been individualized, and recognized as dendritic cells, while two major population of monocytes were also characterized. The first DC population represents ~0.5% of PBMC (5% of monocytic cells) in human, and corresponds to classical blood DCs (BDC) [100]. These blood DC stimulate T-cell

proliferation *in vitro*, express Class II antigens and CD11c, and are negative for the monocyte markers CD14 and CD16. In human, among these blood DC a major subset expresses CD1c, and a minor subset expresses BDCA3 [101]. CD1c is also expressed by B-cells. These classical blood DC are extremely rare in the blood of mice kept in SPF facilities (F. Geissmann, unpublished observation). The second DC subset corresponds to plasmacytoid dendritic cells (PDC), and also represents approximately 0.5% of PBMC in human. PDC have a round shape and a plasmacytoid appearance, and have been initially described as plasmacytoid monocytes, or plasmacytoid lymphocytes [102, 103]. They have been characterized by their capacity to produce high levels of type I Interferon in response to nucleic acids, through TLR8 and TLR9. Indeed, PDCs are the most potent IFN-alpha-producing cells in response to viral pathogens [102, 104]. In human, PDC express HLA class II antigens, are CD123^{high}, and selectively express BDCA2 and BDCA4 (also known as neuropilin 1), but do not express CD11c, CD11b, CD14 or CD16 [105]. In mice, PDC express B220, CD11c^{int}, Class II antigens, while monocytes are negative for these markers.

14.3.2
Monocyte Subsets

The remaining 90% of monocytic cells are, at the present time, considered by the majority of authors as *bone fide* monocytes [9, 11, 19, 99, 106, 107]. In humans and mice monocytes have some typical morphological features such as irregular cell shape, oval- or kidney-shaped nucleus, cytoplasmic vesicles, and high cytoplasm-to-nucleus ratio. However, they are still very heterogeneous in size and shape, in the abundance and morphology of cytoplasmic organelles, and also in their phenotype. As such they may be difficult to distinguish by morphology or by light scatter analysis alone from activated lymphocytes and NK cells. They are best identified by flow cytometry based on forward and size scatter characteristics plus a combination of membrane markers. For example, human monocytes are identified based on their FSC SSC profile and the expression of class II antigens, CD11c, CD11b, and of CD14 or CD16 antigens [99, 106]. Mouse monocytes, in contrast to human monocytes, do not express class II antigens and CD11c, and are identified in blood based on their FSC SSC profile and the expression of F4/80, CD11b, Dectin-1 (the beta glucan receptor), and the 7/4 antigen [108].

During the past few years several mouse models have been generated that may be useful for studying *in vivo* the differentiation and recruitment of blood monocytes, by inserting GFP into the *Lysozyme-M* gene [109], or into the *Cx$_3$cr1* gene [110] or as a transgene driven by the *c-fms* promoter [111]. All circulating CD11b$^+$ F4/80$^+$ monocytes express the GFP reporter in *Cx$_3$cr1*$^{gfp/+}$ mice [110] and several teams have studied blood monocytes in some detail in this model [11, 112].

As a results of *in vivo* studies in mouse models, and *in vitro* studies in several other species, it is now recognized that mouse and human, but also pig and sheep blood monocytes can be divided into two main subsets, with distinct morphology and phenotype (see Fig. 14.2), which express a different set of chemokine re-

ceptors, and which may exert distinct functions [11, 19, 34, 98, 99, 107, 113, 114]. The human subsets have been initially described by the group of Loems Ziegler-Heilbrock [98].

The two mouse subsets (Gr1$^+$ CCR2$^+$ CX3CR1^{lo} and Gr1$^-$ CCR2$^-$ CX3CR1^{high}) share similar expression of M-CSF receptor (CD115), CD11b, and F4/80, and de-

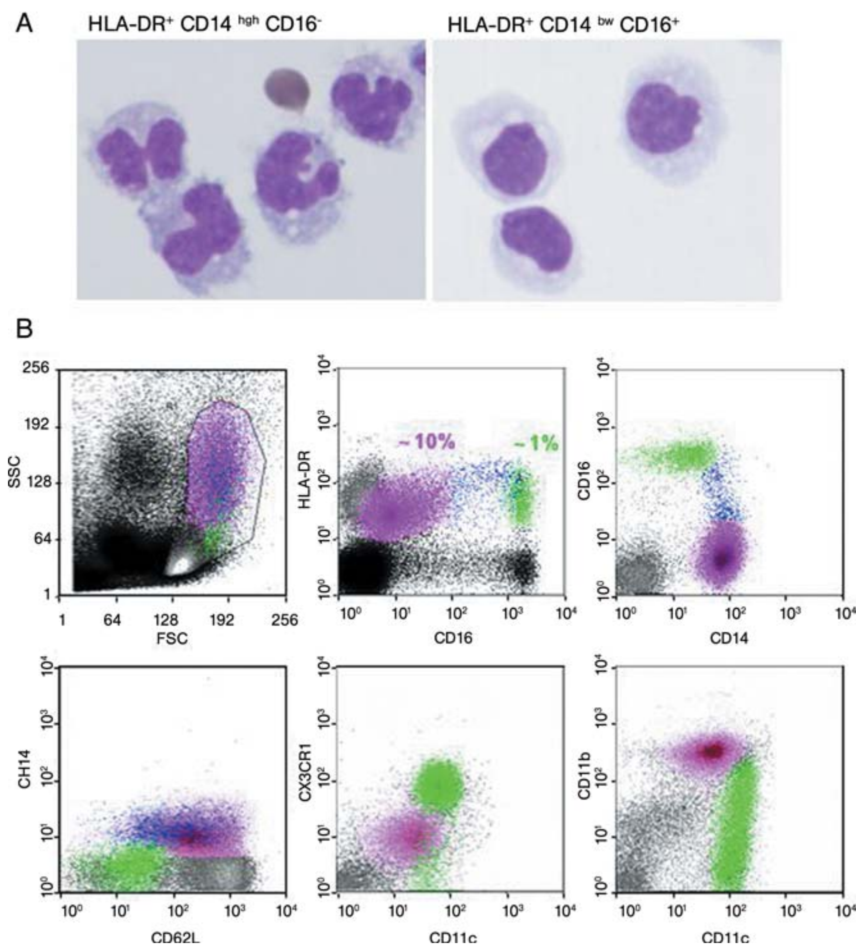

Fig. 14.2 Morphology and phenotype of human monocyte subsets. A. May-grunwald-Giemsa staining of sorted HLA-DR$^+$ CD14$^+$ CD16$^-$ monocytes (left panel), and HLA-DR$^+$ CD14$^-$ CD16$^+$ monocytes (right panel). B. Size, granularity, and surface phenotype of HLADR$^+$ CD14$^+$ CD16$^-$ monocytes (gated in pink color), HLA-DR$^+$ CD14$^-$ CD16$^+$ monocytes (gated I, green color), and of the HLA-DR$^+$ CD14$^+$ CD16$^+$ population (gated in blue). CD14$^-$ CD16$^+$ and CD14$^+$ CD16$^+$ monocytes exhibit striking differences in the shape and size of their cytoplasm and nuclei, and of their phenotype. Note that the HLA-DR$^+$ CD14$^+$ CD16$^+$ cells have FSC/SSC characteristics and phenotype similar to that of CD14$^+$ CD16$^-$ monocytes.

velopment into macrophages upon M-CSF stimulation and into dendritic cells upon GM-CSF stimulation *in vitro* [11, 19, 112]. The two corresponding subsets of human monocytes ($CD14^+$ $CD16^-$ $CCR2^+$ $CX3CR1^{lo}$ and $CD14^{low}$ $CD16^+$ $CCR2^-$ $CX3CR1^{high}$) also appear to differentiate in DC in the presence of GM-CSF and IL-4 [115, 116]. However, under physiological stimuli, mouse monocytes subsets, as well as human monocytes subsets may have distinct differentiation potential. For example the $CD14^{low}$ $CD16^+$ subset of human monocytes is more efficient than the $CD14^+$ $CD16^-$ in differentiating into dendritic cells in a model tissue setting, in the absence of added cytokines [113].

In contrast to the recent progress in the mouse models, mainly due to the availability of reporter mice as described above, very few studies have dealt with the respective functions of monocytes subsets in human. One reason is that they are difficult to purify. A large volume of blood is required to isolate significant numbers of cells, and commercially available kits do not yet allow the separation of $CD14^+$ $CD16^-$ from $CD14^{low}$ $CD16^+$ cells with an acceptable purity for molecular analysis and cell differentiation studies. Therefore at the present time cell sorting by flow cytometry is required, but must be performed under low pressure to avoid cell activation and cell death.

14.3.3
$CCR2^+$ $CX3CR1^{low}$ "Inflammatory" Monocytes

Using the *Cx_3cr1* reporter mice, we have characterized the phenotype, migration potential, and to some extent the differentiation potential of these two monocyte subsets *in vivo* using adoptive transfer in congenic mice. The first monocyte subset represents 50% of mouse blood monocytes, and is composed of large mononuclear cells that express the Ly6C antigen, recognized by Gr1 antibody (which also recognizes the the Ly6G antigen on PMNs) and by specific Ly6C antibodies. These monocytes express CD11b, F4/80, the chemokine receptor CCR2, L-Selectin (CD62L), and weakly express the chemokine receptor CX3CR1 [11, 112]. After intravenous transfer, they have a short half-life (<24 h) in blood and tissues of the recipient in the absence of inflammation. However, these cells are actively recruited into inflammatory sites such as inflamed peritoneum [11, 19] and inflamed lymph node [112], and may be the precursors for TNF and iNOs producing DCs (Tip-DC) [18]. The chemokine receptor CCR2 and its ligand, the chemokine MCP1, are candidates for mediating this recruitment [18].

Within the inflamed peritoneum, these $CD11c^-$ IA^- $Gr1^+$ $CCR2^+$ $CX3CR1^{lo}$ monocytes can differentiate whithin a few hours into CD11c+ IA+ dendritic cells and into $CD11c^-$ IA^- macrophages. The $CD11c^+$ IA^+ DC are able to migrate to the draining parathymic lymph nodes, and can reconstitute the ability of class I KO recipients to trigger the proliferation of naïve OT-1 antigen-specific CD8 T-cells [11]. $Gr1^+$ $CCR2^+$ $CX3CR1^{lo}$ monocytes therefore can differentiate into macrophages and mature dendritic cells in an inflammatory environment.

Human $CD14^+$ monocytes have a phenotype similar to that of the mouse $Gr1^+$ $CCR2^+$ $CX3CR1^{lo}$ monocytes. $CD14^+$ monocytes are large cells that express

CD11b, inflammatory chemokine receptors such as CCR2 and L-Selectin CD62L but low level of CX3CR1 (Fig. 14.2) [11, 107, 117]. In contrast to mouse monocytes, human monocytes also express MHC II antigens and CD11c. Human CD14^{+} CCR2^{+} CX3CR1^{lo} monocytes represent 90% of peripheral blood monocytes. Based on RT-PCR analysis, CD14^{+} CCR2^{+} CX3CR1^{lo} monocytes but not CD16^{+} monocytes (see below), have been reported to produce IL10 after treatment with LPS [118]. A similar observation was made in the pig where the CD163^{-} monocytes, the putative equivalent of the mouse and human CCR2^{+} CX3CR1^{lo} monocytes, produce IL10 mRNA while the CD163^{+} subset, the putative equivalent to the CCR2^{-} CX3CR1^{high} monocytes does not [114].

14.3.4 The Enigmatic CCR2^{-} CX3CR1^{high} Monocytes

The second mouse monocyte subset also represents 50% of mouse blood monocytes, and is composed of smaller cells that also express CD11b (although at a lower level than CCR2^{+} CX3CR1^{low} monocytes), CD115, and F4/80, but does not express Gr1 (Ly6C), CCR2 and CD62L, and expresses high levels of CX3CR1. These Gr1^{-} CCR2^{-} CX3CR1^{hi} cells recirculate in the peripheral blood after adoptive transfer and extravasate into the spleen, lung, liver, and brain, where they persist for several days. Of note, Gr1^{-} CCR2^{-} CX3CR1^{hi} monocytes can also be recruited to sites of inflammation. Recruitment of CX3CR1^{hi} monocytes to non-inflamed peripheral tissues is pertussis-toxin sensitive. Competition experiments between CX3CR1$^{+/-}$ and CX3CR1$^{-/-}$ cells suggested that CX3CR1 plays an important role in the persistence of CX3CR1^{hi} monocytes in the blood and peripheral tissues. However, the nature of the cell(s) that differentiate from CX3CR1^{hi} monocytes within tissues is not yet determined.

These murine CCR2^{-} CX3CR1^{hi} monocytes have a phenotype reminiscent that of human CD14^{low} CD16^{+} monocytes. Human CD14^{low} CD16^{+} monocytes are smaller than the CD14^{+} CD16^{-} monocytes, they lack inflammatory chemokine receptors such as CCR2, and CD62L expression, but strongly express CX3CR1 [11, 98, 107, 117]. Like the murine CCR2^{-} CX3CR1^{hi} cells, they express lower level of CD11b than CCR2^{+} CX3CR1^{low} monocytes. Human CD14^{low} CD16^{+} CCR2^{-} CX3CR1^{high} monocytes express higher CD11c and MHC Class II than CD14^{+} CCR2^{+} CX3CR1^{low} monocytes (see Fig. 14.2).

Human CD14^{low} CD16^{+} represent only 10% of human blood monocytes, but they are more abundant in the blood of patients with sepsis, human immunodeficiency virus 1 infection, and cancer [119]. *In vitro*, human CD14^{low} CD16^{+} are predisposed to become migratory DCs in an reverse transmigration assay [113], and Randolph and his collaborators have thus proposed that human CD14^{low} CD16^{+} monocytes may contribute significantly to precursors for DCs that transiently survey tissues and migrate to lymph nodes via afferent lymphatic vessels.

CD14^{low} CD16^{+} monocytes appear to be the main blood cell type producer of TNFα in response to LPS *in vitro*, at least in human [120]. Interestingly, CD14^{low} CD16^{+} have also been described as a DC subset, termed M-DC8 DC because of

their expression of the antigen 6-sulfo LacNAc (M-DC8), a carbohydrate modification of the P selectin glycoprotein ligand 1 (PSGL-1) [121–124].

14.3.5 Relationship Between CCR2$^+$ CX3CR1^{low} Monocytes and CCR2$^-$ CX3CR1^{high} Monocytes

It has been proposed that human CD14$^+$ CD16$^-$ CCR2$^+$ CX3CR1^{low} monocytes give rise to CD14^{low} CD16$^+$ CCR2$^-$ CX3CR1^{high} monocytes. Human CD14^{high} monocytes can be induced *in vitro* to express CD16 by cytokines such as M-CSF [125–127] and *in vitro* migratory characteristics of CD16$^+$ monocytes are inducible in CD16^{low} monocytes by preincubation with TGFbeta1 [113]. However, in normal human blood a subset of CCR2$^+$ CX3CR1^{low} CCD14$^+$ monocytes express CD16 (see Fig. 14.2), and we have observed that this CD14$^+$ CD16$^+$ subset expands in patients with inflammatory disease, but remain distinct from the CCR2$^-$ CX3CR1^{high} CD14^{low} CD16$^+$ monocytes (our unpublished results). Therefore, up-regulation of CD16 by CD14$^+$ monocyte does not necessarily mean that CD14$^+$ CD16$^-$ CCR2$^+$ CX3CR1^{low} gives rise to CD14^{low} CD16$^+$ CCR2$^-$ CX3CR1^{high} monocytes.

Findings from Sunderkotter et al., also support a developmental relationship between the murine equivalents, Gr1$^+$ CCR2$^+$ CX3CR1^{low} and Gr1$^-$ CCR2$^-$ CX3CR1^{high} subsets. They also propose that Gr1$^+$ CCR2$^+$ CX3CR1^{low} monocytes mature in the circulation and are the precursors for Gr1$^-$ CCR2$^-$ CX3CR1^{high} monocytes. They eliminated blood monocytes with dichloromethylene-bisphosphonate-loaded liposomes and then monitored their repopulation. They observed that monocytes expressing Gr1 first reappeared in the circulation, and that at later time point Gr1 expression on these cells appeared to be downregulated [19]. However, in attempts to approach this maturation sequence more directly after adoptive transfer of purified Gr1$^+$ CCR2$^+$ CX3CR1^{low} monocytes, we, as well as Sunderkotter et al., did not observe the appearance of purified Gr1$^-$ CCR2$^-$ CX3CR1^{high} monocytes in the blood of the recipients in the absence of inflammation, while we were able to track their recruitment to inflamed tissues [11, 19]. Therefore, the relationship between CCR2$^+$ CX3CR1^{low} monocytes and CCR2$^-$ CX3CR1^{high} monocytes remains controversial.

14.3.6 Additional Subsets of Monocytes?

In a recent report, a new (minor) population of Gr1^{intermediate} mouse monocytes, was found to co-express CCR2 CX_3CR1 CCR8 and CCR7 and was also shown to be responsible for the engulfement of Latex microspheres injected i.c. into back skin of mice and their transport in the draining lymph node [128]. Unexpectedly neither CCR2 nor CX3CR1 were required [128] while CCR2 appears to be required for the recruitment and/or differentiation of the precursors for the TipDCs in the spleen [18, 76]. The relationship between Gr1^{intermediate} monocytes and the two Gr1$^+$ CCR2$^+$ CX3CR1^{low} and Gr1$^-$ CCR2$^-$ CX3CR1^{high} monocytes described here should be investigated.

A population of human monocytes responsive to the chemokine BRAK (CXCL14) has been also reported (see section 14.4) [129], but it is not yet known whether they belong to the CCR2^{+} CX3CR1^{low}, or CCR2^{-} CX3CR1^{high} subset or if they represent an additional population of monocytes. It will be interesting to investigate whether or not resident monocytes can be further divided into functional subsets that home to distinct sites, e.g., skin, bone, lung, or brain, or that exhibit different responses to pathogens, and may, for example, differ in the expression or pattern recognition receptors such as scavenger receptors and Toll-like receptors.

14.4 Migration of Monocytes and Their Recruitment to Tissues

14.4.1 Monocyte Entry into the Target Tissues

A critical step in monocyte differentiation into macrophages and dendritic cell subsets is their entry into the target tissues. Half of the circulating monocytes leave the bloodstream under steady-state conditions each day in mice [130–133]. In humans the half-life of circulating monocytes is about three times longer than in mice but they represent 10% of blood mononuclear cells instead of 3% in the mice, and the monocyte mass is a thousandfold greater in humans than in mice [134]. A great deal of research has identified chemoattractants and cellular activators responsible for lymphocyte and neutrophil trafficking into inflamed tissues, as well as for lymphocyte homing to secondary lymphoid organs in the steady state and into foci of chronic inflammation. Considerably less is known about the molecules regulating the trafficking of monocytes, particularly the constitutive trafficking of monocytes through tissues in the steady state, and the recruitment of monocytes to lymph nodes and inflamed tissues in disease.

Blood circulating monocytes are distributed over a circulating and a marginating pool. In mice, this circulating pool accounts for approximately 40%, and the marginating pool for approximately 60% of the population of peripheral blood monocytes [135]. This marginating pool may correspond mostly to CD16^{+} monocytes rolling on the endothelium, and that can be mobilized in human in response to stress or physical exercise [136]. Rolling can also precede extravasation and monocytes have indeed been shown to extravasate across the endothelial barrier *in vitro* and *in vivo*, usually in response to inflammatory stimuli, but also independently of inflammation, and to differentiate into macrophage-like and dendritic-like cells [11, 17, 27]. Chemokine receptors/ chemokine pairs, such as CCR2/MCP-1 and CX3CR1/Frackalkine, most likely play important roles in the adhesion of monocytes to endothelium and in extravasation [107, 137].

Recent studies have investigated the mechanisms of diapedesis, the forward migration of leukocytes through endothelial junctions, by monocytes. During the extravasation process, blockade of CD11a-CD18 and CD11b-CD18 on human monocytes or adhesion molecules ICAM-1 and ICAM-2 on endothelial cells prevents the monocytes from reaching junctions and thus from performing diapedesis [138].

Diapedesisis is then regulated sequentially by two distinct molecules, PECAM-1 and CD99 [139].

14.4.2
Baseline Extravasation of Monocytes

Recruitment of monocytes to peripheral tissues is pertussis-toxin sensitive [11], suggesting the implication of chemokine receptors. Competition experiments between $Cx_3cr1^{+/-}$ and $Cx_3cr1^{-/-}$ cells *in vivo* showed that $Cx_3cr1^{-/-}$ Gr1$^-$ CCR2$^-$ monocytes failed to accumulate in tissues after transfer, which may point to a role for CX3CR1 in the extravasation of these monocytes in the steady state, but $Cx3cr1^{-/-}$ Gr1$^-$ CCR2$^-$ monocytes also failed to accumulate in the blood, and may thus have a decreased survival *in vivo*. The former hypothesis is supported by a study on the corresponding human CD14^{low} CD16$^+$ CCR2$^-$ CX3CR1^{high} monocyte population, which showed that in contrast to CD14$^+$ CD16$^-$ CCR2$^+$ CX3CR1^{low}, CD16$^+$ monocytes underwent efficient transendothelial migration in response to fractalkine (FKN; FKN/CX3CL1) [107]. Murine Gr1$^-$ CCR2$^-$ CX3CR1^{high} monocytes and human CD16$^+$ CCR2$^-$ CX3CR1^{high} monocytes also express CXCR4 [11, 107], and CD16$^+$ CCR2$^-$ CX3CR1^{high} monocytes were shown to undergo transendothelial migration *in vitro* in response to stromal-derived factor 1 alpha (SDF1, CXCL12) the ligand for CXCR4.

Breast and kidney expressed chemokine (BRAK, CXCL14) mRNA is expressed constitutively by a variety of epithelia including the basal keratinocytes and dermal fibroblasts of skin, and cells in the lamina propria of gut [129, 140]. A fraction of monocytes present in peripheral blood (see Section 13.3.6) was found to be responsive to BRAK in the absence of exogenous activation, and therefore the work of Kurth et al. [129] suggests a possible role for BRAK in the constitutive recruitment of this subpopulation of monocytes into BRAK-expressing tissues such as subepithelial locations by BRAK bound to and presented by endothelial cell heparan sulfate. This would allow monocytes, but not lymphocytes or neutrophils, to leave the circulation and enter these tissues in the absence of inflammation. The receptor for BRAK is not known, and it is not known if monocytes responsive to BRAK belong to the CD16$^+$ CCR2$^-$ CX3CR1^{high} monocytes or CD14$^+$ CCR2$^+$ CX3CR1^{low} subset. Monocyte migration in response to BRAK is regulated. Under baseline conditions *in vitro* only a fraction of monocytes migrate in response to BRAK, but as many as 20% of monocytes migrate in response to BRAK after activation by prostaglandin E2 (PGE2) [129]. Monocytes respond to BRAK in a pertussis toxin-sensitive manner. The fate of monocytes that have entered tissues in response to BRAK is unknown.

14.4.3
Recruiment of Monocytes During Inflammation and Infection

In mice responding to an inflammatory challenge, the number of monocytes leaving the circulation per day is at least double. Monocytes are actively recruited from

the blood during inflammation and are believed to play a crucial role in numerous pathological processes such as infection or immune-mediated disease such as arteriosclerosis.

The hallmark of early atherosclerosis is the accumulation of lipid-laden macrophages in the subendothelial space. Circulating monocytes are the precursors of these "foam cells," and recent evidence suggests that chemokines such as CCR2 and CX3CR1 play important roles in directing monocyte migration from the blood to the vessel wall and to macrophage recruitment in atherosclerotic lesions. Cx3cr1$^{-/-}$ apoE$^{-/-}$ mice have a significant reduction in macrophage recruitment to the vessel wall and decreased atherosclerotic lesion formation [141, 142], and a polymorphism in cx3cr1 has been linked to a decrease in the incidence of coronary artery disease in human [143]. CCR2$^{-/-}$, apoE$^{-/-}$ mice also showed a 50% reduction in macrophage recruitment to atherosclerotic lesion compared with wild type after 5 wk on a Western diet [144].

The chemokine receptor CCR2 is crucial for recruiting monocytes/macrophages to sites of inflammation and infection [73, 137]. During infection, recent evidence discussed above suggest that monocytes from the inflammatory CCR2^{+} CX3CR1^{low} Gr1^{+} subset use CCR2 to enter inflamed spleen during *Listeria monocytogenes* systemic infection and the site of infection during acute toxoplasmosis [18, 76]. In toxoplasmosis and listeriosis, the failure to recruit CCR2^{+} CX3CR1^{low} Gr1^{+} monocytes was associated to a greatly enhanced mortality despite the induction of normal T-cel responses. The profound susceptibility of CCR2$^{-/-}$ mice suggests that CCR2^{+} CX3CR1^{low} Gr1^{+} monocytes are important effector cells in the resistance to listeriois and acute toxoplasmosis and suggests that the CCR2-dependent recruitment of CCR2^{+} CX3CR1^{low} Gr1^{+} monocytes may be an important general mechanism for resistance to intracellular pathogens.

14.5 Concluding Remarks

Monocyte differentiation has been an area of intense investigations for the past 10 years. The recent development of new tools and protocols has allowed these studies to be extended to *in vivo* models. These *in vivo* studies have uncovered the existence of functional subsets. Recent studies investigating the physiological regulation of monocyte differentiation have begun to modify the current views on the differentiation potential of monocytes. More studies are under way in several laboratories, and results from these experiments may well differ significantly from those observed upon cytokine treatment of monocytes *in vitro*. It will therefore now be feasible to distinguish the respective functions of monocytes subsets, their develepemental relationship, and their respective contributions to the renewal of distinct subsets of cells of the mononuclear phagocytes system, in physiological and in pathological conditions.

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V
Dendritic Cell Migration

15
Steady State Migration of Dendritic Cells in Lymph

Gordon MacPherson, Simon Milling, Emma Turnbull and Ulf Yrlid

15.1
Introduction

15.1.1
Dendritic Cells

Dendritic cells (DCs) are crucial antigen-presenting cells (APCs) in the initiation of immune responses. They are not to be confused with follicular dendritic cells (FDCs) which are very long-lived, non-hemopoietic, resident cells in B-cell areas of secondary lymphoid organs and which retain antigen–antibody complexes on their plasma membranes for long periods. DCs are bone marrow-derived cells that enter peripheral tissues from blood, spend varying periods in tissues, acquire antigens (Ag) (both self and foreign) and migrate from the periphery via afferent lymphatics to lymph nodes. In the node they present Ags to recirculating T and probably B lymphocytes, deliver signals essential for activation of naïve T cells and influence the differentiation of the lymphocytes they activate. They subsequently die in the node, presumably by apoptosis. A crucial concept is that this cycle goes on continually in the steady state in the absence of any apparent inflammatory "danger" signals. As well as their importance in the activation of lymphocytes, there is increasing evidence for their involvement in T-cell tolerization and the induction of regulatory T cells.

The ability to migrate is central to the function of DCs and the study of DC migratory processes has given many valuable insights into their biology. This review will focus on studies of DCs migrating in lymph and will relate these to other methods that have contributed to the understanding of DC biology. It will concentrate on our own studies in the rat but will attempt to relate these findings to studies of DC migration in other species. We would draw the reader's attention to some other recent reviews that concentrate on rat DCs [1, 2] and on lymph DCs (LDCs) [3].

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15.1.2
Why Study Lymph Dendritic Cells?

DCs are difficult cells to study. They are rare cell types in both peripheral tissues and lymphoid organs and their extraction generally requires enzymatic digestion (we have found that nonenzymatic isolation of DCs from rat spleen results in much reduced yields [MacPherson and Jenkins, unpublished observations]), usually with collagenase and DNAse, followed by separation using combinations of density gradient centrifugation and flow cytometric or magnetic bead sorting. DCs are very sensitive to environmental changes; indeed their ability to respond to these changes is probably central to their functions in immune regulation and it is now clear that lengthy separation procedures induce changes in DC phenotype and function that are usually associated with maturation. Another difficulty in interpreting studies involving DCs isolated from solid tissues is that the isolated population may not be representative of the population *in situ*. There may for example be selective extraction of certain DC subsets. In this context it is at present impossible to estimate quantitatively the fraction of total DCs that is recovered from tissues; we do not know if it is 0.1%, 1% or 10%. Our impression from studies in that rat is that it more likely to be in the region of 1% from the small intestine, thus greatly increasing the possibility of bias in subset isolation.

A further problem with isolation of DCs from solid tissues is that the DCs in those tissues, both lymphoid and nonlymphoid, represent cells at different stages of their life history. In the gut for instance, where DCs spend on average only 3–4 days, the extracted DCs will represent recent blood emigrants, cells actively engaged in antigen acquisition and those on the point of exit into lymph.

The study of LDCs overcomes several but not all of these problems. Most importantly, that these DC can be collected and maintained at 4oC moments after they exit the tissue ensures that their phenotype and patterns of gene expression will not have changed significantly and thus these cells represent as close to the *in vivo* state as can be achieved. Additionally, these DCs are those actively involved in antigen transport and are those that will, on entering their draining lymph node, help to regulate the differentiation pathways of both T and B lymphocytes that they encounter and activate.

15.1.3
Lymphatic Terminology

All lymph nodes have afferent lymphatic inputs and efferent lymphatic outputs. Lymph nodes however often exist in chains and thus afferent lymph differs depending on whether or not the lymph has previously passed through a node. Afferent lymph can be divided into peripheral lymph, which is directly draining a tissue and has not passed through a node, and intermediate lymph which has passed through at least one node. Throughout this review, when afferent lymph is mentioned, we imply peripheral lymph.

15.1.4 Historical

The first studies examining the cellular content of peripheral (afferent) lymph revealed that the presence of a nonlymphocytic population. On the basis of their morphology, these cells were assumed to be macrophages. It is now clear that steady-state afferent lymph does not contain significant numbers of classical macrophages or monocytes, and that the cells described are in fact DCs. This area is reviewed in some detail in Ref. [4], but as this volume is not widely available we will summarize the major findings here. The first systematic study of the cellular content of afferent lymph was made by Yoffey and Drinker in 1939 (reviewed in Ref. [4]). They observed that about 20% of cells from limb lymph were "macrophages". The most intensive early studies were made in sheep by Bede Morris and his collaborators in Canberra. In an elegant series of studies [5–10] they examined peripheral lymph draining many organs and tissues including skin, testis, ovary, kidney, liver and thyroid. In general they found that the numbers of "macrophages" exiting these sites were similar, comprising 5–20% of the total cell output. They also noted that although the proportion of "macrophages" in hepatic lymph was similar to that from other tissues, as the total cell output from the liver was 4–6-fold higher, the "macrophage" output was numerically much greater than from other tissues. These cells were considered to be Kupffer cells released from the liver sinusoids. This problem was only resolved recently by work from Matsuno [11] who described a novel DC migratory pathway from hepatic sinusoids to the celiac node. All of the earlier studies however viewed these cells as macrophages and it was not until others, in particular Balfour, Drexhage and Knight, studying peripheral lymph in rabbits and pigs [12–16], and Mayrhofer [17] and our group [18–22] using a rat model, showed that lymph-borne veiled cells had many properties in common with the recently-discovered DC [23–28].

15.2 Dendritic Cells in the Periphery

15.2.1 Constitutive Migration of Dendritic Cells from Peripheral Tissues

It is widely believed that DCs represent a static population in the periphery, residing in tissues until they receive a stimulus (typically inflammation or other tissue damage), which induces them to mature and enter lymphatics (for example see Ref. [29]). If this were the case then we would expect lymph draining unperturbed tissues and organs to be DC-free. This is clearly not the case. DCs are found in all afferent and pseudo-afferent lymph that has been examined [9, 10, 30–35]. In the case of cows and sheep, pseudo-afferent lymph flow can be maintained for days or weeks after surgery [30, 31, 35–40]. DC turnover in lymph, peripheral tissues and nodes has been studied in rats by us [19, 41], Holt [42, 43] and Fossum [44] and in mice [24, 45–47]. In all these cases, DC lifespan in tissues and secondary lymphoid

tissues is measured in days, suggesting a rapid turnover. It is difficult to construct models in which pools of DCs are turning over independently in peripheral tissues and lymph nodes, particularly so if DCs migrating in lymph, which are also short-lived, have to be taken into account. However, one population of DCs are an exception to this rule. Langerhans cells display very different kinetics compared with DCs from other tissues. They are much longer-lived than other DC populations and are a self-replenishing population under steady state conditions, rather than derived from bone marrow precursors [48, 49]. The general conclusion is that DCs are continually migrating from peripheral tissues into lymph and then to nodes in the absence of tissue damage. That this migration may be crucial for the induction of tolerance to self- and non-harmful foreign antigens is supported by studies showing that some DC subpopulations are able to present antigens in a manner that leads to T-cell tolerance [50–52].

15.2.2
Exit of Dendritic Cells from Peripheral Tissues

It is generally accepted that the major exit route for DCs leaving tissues is via peripheral lymph. Under some circumstances however, DCs may enter blood directly from tissues, as has been suggested by Austyn for solid organs [53–57]. Thus, anterior chamber immune deviation is dependent on the migration of an antigen-bearing cell with the properties of an immature DC, from the eye via blood to the spleen [58, 59], while orally administered invasion-deficient *Salmonella* are rapidly detectable within blood cells that display DC properties [60].

15.2.3
Entry of Dendritic Cells into Peripheral Lymph

This topic has been reviewed recently by Randolph [61], and will not be covered in detail here. Suffice it to say that almost all studies investigating the factors regulating DC egress from tissues have employed models where DC migration has been stimulated in one way or another. Virtually nothing is known about the regulation of DC egress under steady-state conditions. It is however unlikely to represent a completely random process as DC migrating in intestinal lymph are phenotypically distinct from the bulk of those present in the intestine itself [62], see below.

15.3
Lymph Dendritic Cells

15.3.1
Pseudo-afferent Lymph

DCs migrate in peripheral lymph to lymph nodes but most (90–95% at least) are filtered out in the node [63]. Under steady-state conditions, DCs are difficult to de-

tect in efferent lymph. It is possible to cannulate afferent lymphatics directly in the rat [19] but the volumes of lymph obtainable are tiny. Sanders and Florey showed that it was possible to remove lymph nodes surgically in the rat without obvious detriment. Graham Mayrhofer, working with Gowans, then showed that following mesenteric lymphadenectomy (MLNX), afferent and efferent lymphatics joined together, permitting cells derived from the intestine to migrate directly into the thoracic duct (Mayrhofer, personal communication). Techniques for thoracic duct cannulation were well-established ([64] and had been utilized by Gowans for studies of lymphocyte recirculation [65]. Thus these approaches, later used in several other species, permitted direct collection of cells, including DCs, which had recently left the intestine. This approach has been used by Matsuno [11, 63, 66, 67] and more recently by ourselves to study liver DCs after removal of the hepatic nodes, and by Mayrhofer to study hindquarter lymph after removal of the chain of nodes draining these tissues (Mayrhofer, personal communication). The technique has also been adapted to cows [3, 34, 35, 38, 39, 68–73] and sheep [30–32, 36].

15.4 Properties of Lymph Dendritic Cells in the Rat

15.4.1 Steady-state Output

DCs enter rat intestinal pseudo-afferent lymph at a relatively a constant rate for at least 5 days after cannulation [19] suggesting that DC output is not altered by the acute effects of surgery. In sheep and cows, where long-term cannulation is possible, DC output into lymph is maintained at a relatively constant level for at least 2 weeks. Pro-inflammatory stimuli can certainly stimulate increased DC output into lymph [62, 74] (see below), but the steady-state migration is substantial, constitutive and is presumed to have a functional role, perhaps in the induction and maintenance of tolerance to peripheral self antigens and harmless exogenous antigens (e.g. food and commensal bacteria).

15.4.2 Origin of Afferent Lymph Dendritic Cells

The mesenteric nodes are the first nodes encountered by peripheral lymph from the small intestine, caecum and upper colon (described in a definitive paper by Tilney [75]). Thus DCs present in pseudo-afferent lymph following MLNX could derive from any of these sources.

15.4.3
Steady-state Rat Lymph Dendritic Cells are "Semi-mature"

Intestinal DCs in the rat are identified by expression of CD11c, MHC class II and CD103 (recognized by the OX62 antibody [76]). DCs *in situ* in the lamina propria have immunocytochemical properties characteristic of typical immature DCs. In particular the bulk of MHC class II is in intracellular vesicles [62]. DCs isolated from the lamina propria are weak stimulators of an allogeneic mixed leukocyte reaction (MLR) [77], but do contain a small population expressing high levels of surface MHC class II (Yrlid and MacPherson, unpublished observations).

In contrast, essentially all intestinal lymph DCs express high levels of surface MHC class II, with very little being intracellular [62], and these DCs are the most potent stimulators of an allogeneic MLR we have found, being considerably stronger than spleen DCs (Yrlid and MacPherson, unpublished observations). Surface co-stimulatory molecule expression is however very weak, with essentially no CD80 and only very low levels of CD86 and CD40 being present. These lymph DCs are thus similar to the "semi-mature" DC described in murine bone marrow cultured DCs by Lutz [78].

15.4.4
Subsets of Rat Lymph Dendritic Cells

We can identify DCs in pseudo-afferent intestinal lymph by their co-expression of MHC class II, CD11c, and CD103. We observed that these DCs, examined as living cells or on cytospins, expressed two distinct morphologies. Some had many short plasma membrane spikes whereas other larger cells expressed a few long blunt pseudopodia. Expression of CD172 (SIRP-α), recognized by the OX41 mAb, divides lymph DCs into two subsets, one labeled much more strongly than the other. The CD172^{high} subset also expresses CD4 and represent the "spiky" DCs. In steady-state lymph about 60% of DCs are CD172^{high}. Interestingly the CD172^{high} and CD172^{lo} subsets cannot be distinguished by their expression of MHC class II and co-stimulatory molecules [62, 79]. Expression of CD90 (Thy1) is higher on the CD172^{high} DCs (but all DCs become CD90+ after overnight culture). The origins of these DC subpopulations are nor clear. We do not think that one population is the precursor of the other because following i.v. BrdU administration, BrdU-labeled CD172^{hi} and CD172^{lo} DCs enter pseudo-afferent intestinal lymph with similar kinetics [79]. (Whether they are derived from distinct precursors or whether a single precursor can differentiate into both subsets is unknown at present. It is of interest that DCs derived from rat bone marrow cells cultured with GM-CSF and IL-4 differ in several respects from both of the lymph DC subsets. Lymph DCs are significantly more potent stimulators of an allogeneic MLR and whereas BMDC can be stimulated to secrete large amounts of nitric oxide, lymph DCs cannot be stimulated to secrete any, or to express nitric oxide synthase [80]). In contrast, preliminary experiments suggest that BMDC obtained by culture with FLT-3 ligand contain CD172^{high} and ^{lo} subsets and which are CD103 (Yrlid and MacPherson, un-

published observations) may be more representative of *in vivo* DC populations. Functionally, CD172^{high} and CD172^{low} subsets differ markedly. Lymph DCs in general survive poorly *in vitro*, with less that 20% surviving after overnight culture. Survival of CD172^{high} DCs can be increased markedly by culture with GM-CSF or IL-4, but such treatments have very little effect on CD172^{low} DC survival [21, 22]. Whether this reflects survival *in vivo* of the subsets is not known but preliminary data suggests that some CD172^{lo} DC are already in early apoptosis by the time they enter lymph (Yrlid and MacPherson, unpublished data).

In all assays of T-cell activation, CD172^{lo} DCs are weaker APC than their CD172^{high} counterparts. This is true of stimulation of an allogeneic MLR, presentation of antigen to sensitized T cells *in vitro* and sensitization of naïve T cells *in vivo* [41], although we cannot be sure whether the differential survival of these subsets is contributing in these differences in function.

It is widely thought that DC maturation is coupled to a downregulated ability to process native antigens [81]. We cultured lymph DCs for different periods, pulsed them with native antigen and assessed their ability to activate sensitized T cells *in vitro*. CD172^{lo} DCs rapidly lost the ability to process native antigen but in contrast, CD172^{high} DCs retained the ability to process and present OVA for at least 72 h in culture [41, 79].

DC heterogeneity in lymph is not confined to the rat. Studies in cows by Howard's group [3, 34, 35, 38, 72, 73, 82], and in sheep by Hopkins' group [30, 32, 36, 83] have identified phenotypically and functionally distinct subsets of migrating DCs that exhibit interesting similarities to rat lymph DCs. How all of these subsets relate to murine DC subsets is unclear, but work from our group and that of Josien would suggest that there are distinct similarities between murine splenic CD8+ DCs and rat splenic CD172^{lo} DCs, and between murine splenic CD8– DCs and rat splenic CD172^{high} DCs in terms of their anatomical localization and functional properties (particularly the uptake of apoptotic cells *in vivo*). It is less clear how subpopulations of DCs in rat lymph relate to murine DCs. In particular all lymph DCs express high levels of MHC class II, whereas in mice, only a subpopulation of lymph node DCs, thought to represent migrants from peripheral tissues, are MHC class II^{high} [84, 85]. It has been suggested that migrating DC in mice are a homogenous population. This is clearly not true in rats, or in cows and sheep, where direct examination of DC in lymph has defined at least two subpopulations in all cases [35, 39–41].

15.4.5
Migratory Fate of Lymph Dendritic Cells

Subpopulations of DCs delineated by CD172 expression are not uniformly distributed in mesenteric nodes. CD172^{lo} DCs are found in the outer cortex, between the B cell follicles, and throughout the T-cell area (paracortex). CD172^{high} DCs are not found throughout the T-cell areas under steady state conditions but are confined to the interfollicular areas, with small numbers being present immediately under the follicles [62]. This region under the follicles, which is adjacent to high-endothelial

venules, has recently been highlighted as an area where there is a dense network of DCs, and may be important in the initial activation of lymphocytes [86]. Similar partitioning of DC subsets has been observed in murine spleen [87].

15.4.6 Uptake and Transport of Apoptotic Cells by Intestinal Dendritic Cells

We observed by light and electron microscopy that a proportion of intestinal lymph DCs contained large cytoplasmic inclusions. Immunocytochemistry showed that these inclusions were present exclusively in CD172^{lo} DCs. We had previously observed that some DCs contained cytoplasmic DNA, as shown by Feulgen staining. Labeling lymph DCs by the Tunel method showed that many of these inclusions contained apoptotic DNA. To ascertain the origin of this apoptotic DNA, lymph DCs were labeled for the presence of an epithelial cell-restricted cytokeratin. A proportion of the CD172^{lo} DCs were found to be cytokeratin+. Furthermore, CD172^{lo} DCs were uniformly strongly positive for nonspecific esterase (NSE). NSE represents a number of different enzymes with differing electrophoretic mobility, and the pattern of bands observed can be characteristic of a particular tissue. The banding pattern of NSE derived from CD172^{lo} DCs was very similar to that seen with intestinal epithelial cells and very different from those seen in other DC or macrophage populations. Only DCs extracted from mesenteric nodes gave bands with mobility similar to lymph DCs or enterocytes. Thus we concluded that CD172^{lo} DCs acquire enterocytes in situ and transport them to mesenteric nodes [88], although quite how DCs acquire enterocytes is unknown. It is likely that they ingest enterocytes which have already undergone apoptosis although we cannot exclude the possibility that the DCs are actively inducing apoptosis. Josien's group has shown that DCs with a similar phenotype in rat spleen can kill cells in a NK cell-like manner [89–91]. The functional significance of transporting apoptotic enterocytes to the draining MLN is not realized yet but it may relate to the maintenance of peripheral tolerance. It is relevant that Germain's group have shown that gastric DC can acquire material from gastric epithelial cells and transport it to draining nodes [92]

15.5 Dendritic Cells and B Cells

In the early stages of an infection, only minimal amounts of antigen will be available to the immune system. It is well-recognized that DCs can act to focus antigen to specific T cells in secondary lymphoid organs. How antigen reaches B cells is however not well understood. It may be by simple diffusion [93, 94], but this would be inefficient under conditions of low antigen concentration. We had observed that DCs freshly isolated from lymph occasionally had lymphocytes adherent to them. On staining cytospin preparations, we were surprised to find that many of these lymphocytes were B cells. To investigate this further we incubated lymph DCs with

fresh, syngeneic, thoracic duct lymphocytes and found that B cells selectively adhered to DCs, forming clusters that lasted for up to 24 h in culture. Cluster formation was energy, cytoskeletal and protein kinase C-dependent, and was stimulated by crosslinking MHC class II, but not other surface molecules on the DC [95]. This interaction suggested that DCs may make functional interactions with B cells. To test this, we investigated the fate of protein antigens in different DC populations. We were surprised to find that enzymatically-active horse radish peroxidase was retained in DCs cultured *in vitro* for at least 48 h [96], and that it appeared to be retained in an intracellular compartment that did not contain MHC class II [96]. To determine if this retention could have functional significance in B cell activation and antibody synthesis, we injected rats i.v. with keyhole limpet hemocyanin (KLH) and recovered splenic DCs at intervals thereafter. These DCs were transferred i.v. into naïve recipients and the presence of anti-KLH antibody assessed at 7 and 14 days. We found that rats given DCs from KLH-injected rats did make antibody, that we could wait for at least 24 h before isolating DCs and still get an antibody response, and that the longer we waited, the more the antibody response in the recipient switched from IgM to IgG. In an *in vitro* model of antibody synthesis using naïve B cells and activated T helper cells, we showed that DCs provided B cells with signals involved in isotype switching and that this signaling was contact-dependent but independent of CD40 [96–98]. More recently, these findings have been confirmed and extended by Mellman's group, who have shown that immature DCs show defective lysosomal acidification and a selective deficit in lysosomal proteases [99–101]. Thus it is becoming apparent that DCs may play an active role in the delivery of intact antigen to B cells and in the initiation of isotype switching.

15.6
Dendritic Cells and the Pathogenesis of Transmissible Spongiform Encephalopathies (TSE)

TSE diseases (scrapie, bovine spongiform encephalopathy etc.) are associated with an abnormal, protease-resistant form of a normal host protein (prion-related protein, PrP). In scrapie, peripheral inoculation of the agent is followed by a period when the agent accumulates/replicates on follicular DCs (FDC) in the B cell follicles of secondary lymphoid tissues. Most BSE and other TSE diseases are thought to be transmitted by the consumption of contaminated food, but how the agent reached secondary lymphoid tissues was unclear. We hypothesized that DCs might acquire the agent from the intestine either via M cells or possibly by the insertion of processes into the intestinal lumen [102–104]. To test this, we injected mesenteric lymphadenectomized rats intra-intestinally with scrapie-associated fibrils (SAF) and examined lymph DCs for the presence of immunoreactive PrP. Between 8 and 24 h after injection, 4–6% of lymph DCs contained conspicuous cytoplasmic PrP [105]. Subsequently we found that unlike macrophages, DCs retained protease-resistant PrP for long periods (at least 72 h). We thus suggest that DCs act as "Trojan Horses", acquiring PrP from the gut and transporting it to mesenteric

nodes where, by unknown mechanisms (involving B cells), it eventually reaches FDC.

15.7 Conclusions

DCs are crucially involved in the initiation and regulation of adaptive immune responses. They also have enormous potential as tools for immunotherapy. They are however difficult cells to study and understand. Much of our understanding of DCs derives from studies of DC derived *in vitro* from bone marrow cells or blood monocytes. If DCs are to be used for immunotherapy, understanding their *in vivo* biology is not crucial if they actually work. Current evidence suggests however that there are many difficulties to surmount if DCs are to realize their potential in therapy. We suggest that it is crucial to understand the immunophysiology of DC *in vivo* if these obstacles are to be overcome. Unraveling the biology of DCs migrating from peripheral tissues to lymph nodes will give insights into these fundamental questions that cannot be achieved using other approaches.

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16
Multiple Pathways to Control DC Migration

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16.1
Dendritic Cells as Professional Migratory Cells

Dendritic cells (DC) are potent antigen presenting cells with a unique ability to induce T- and B-cell responses as well as immune tolerance [1–4]. DC reside in an immature state in peripheral tissues where they exert a sentinel function for incoming antigens [1–4]. Upon microbial contact and stimulation by inflammatory cytokines, DC take up antigens and traffic via the afferent lymphatics into the T-cell area of the draining lymph node to initiate immune responses [2, 5–7]. There is evidence that steady-state migration of DC into the lymph node also occurs in normal conditions and may serve to tolerize T cells against self and non-dangerous antigens [1]. Migration of DC into tissues depends on a cascade of discrete events, including chemokine production and regulation of chemokine receptors [5, 6, 8, 9].

DC are a heterogeneous population that posses unique homing properties [2, 10]. Myeloid blood $CD11c^+$ DC can migrate in response to a wide array of inflammatory chemotactic agonists produced at the peripheral sites of infection and immune reaction [7, 11, 12]. On the other hand, $CD123^+$ plasmacytoid DC are believed to enter lymph nodes across blood high endothelial venules [13]. The expression and regulation of functional chemotactic receptors is likely to be responsible for the different distribution of DC subsets *in vivo*.

The proper localization of DC to secondary lymphoid organs and their recruitment at sites of inflammation in response to chemotactic stimuli are critical events for optimal immune response [14–16]. Recent work has documented that a number of chemotactic agonists, different from chemokines, play a relevant role in DC subset recruitment [12, 17–20]. Furthermore, multiple experimental evidence has shown that chemokine receptor expression is not predictive of DC migration since multiple factors, including prostaglandins, leukotrienes, sphingosine1-phosphate, extracellular nucleotides and some membrane proteins (e.g. CD38) play an important role in the regulation of chemokine receptor function [10, 21–24]. Therefore,

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DC migration *in vivo* is a tightly regulated process controlled at the level of chemokine production and chemokine receptor expression and function.

16.2
Role of Chemokines in the Recruitment of Myeloid and Plasmacytoid Dendritic Cells

Chemokines are small, secreted chemotactic cytokines that regulate the migration of leukocytes under steady state and inflammatory conditions [8, 25, 26]. Immature DC express a unique repertoire of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR5, CCR6) [5, 9, 27]. These receptors bind a pattern of "inflammatory" chemokines, including CCL2, CCL3, CCL4, CCL5, and CCL20 (Fig. 16.1). In addition, immature DC also express functional CXCR4 [28]. Since CXCL12, the CXCR4 ligand, is constitutively expressed in both lymphoid and nonlymphoid tissues, this chemokine may play a role in the recruitment of DC in such tissues.

It is generally believed that DC precursors in the peripheral blood migrate into peripheral tissues and differentiate to become immature DC. Although it is conceivable that the same classes of molecules that regulate DC migration in inflammatory situations also direct the migration of these cells under normal conditions, little is known about the mechanisms that regulate the homing of DC or their precursors, in steady-state conditions [9, 29]. A number of studies have established a role for CCR2 in the localization of Langerhans cell precursors [30–32]. It is of note that transgenic mice over-expressing CCL2, under the keratin promoter, have local accumulation of cells with DC morphology in the basal layer of the epidermis [33]. Following differentiation, Langerhans cell precursors start to express CCR6 and re-

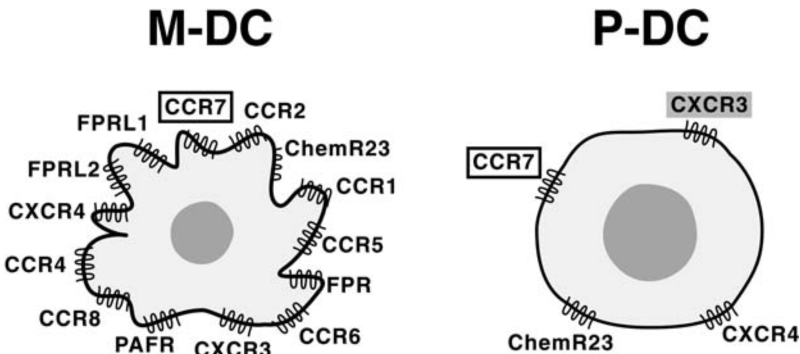

Fig. 16.1 Functional chemokine receptors expressed by myeloid and plasmacytoid dendritic cells. Immature DC express many chemokine receptors for inflammatory chemokines. Maturation induced by inflammatory signals (e.g. LPS, IL-1 and TNF) or immune stimuli (e.g. CD40 ligation) induce DC maturation. Maturation is associated to the downregulation of inflammatory chemokine receptors and the expression of CCR7. CCR7 plays a pivotal function in the migration of DC to draining lymph nodes. Open boxed names indicate expression by mature DC. Shadowed boxed CXCR3 indicates that this receptor is active only when engaged by membrane bound chemokines.

spond to the cognate ligand CCL20 [31, 34, 35]. Since *CCR6*$^{-/-}$ mice have normal numbers of skin DC cells [36], it is likely that the CCR6/CCL20 axis plays a role mainly in inflammatory skin disorders that are characterized by the expression of CCL20 by inflamed keratinocytes [34]. CCL20 plays also a role for the homing of DC to mucosal surfaces. CCL20 is expressed in murine intestinal Peyer's patches, and *CCR6*$^{-/-}$ mice have a defect in humoral immune response to oral antigens [36, 37].

Plasmacytoid DC are a rare subset of cells present in circulation and in secondary lymphoid organs [13, 38]. These cells express MHC class II, have the ability to activate T lymphocytes, and secrete high levels of type I interferon following activation. The production of type I interferon is believed to play a crucial role in antiviral immune responses and in the activation of other leukocyte populations, like B lymphocytes and NK cells [13, 39].

The expression of chemokine receptors on sorted blood myeloid DC and plasmacytoid DC is, in general, fairly similar [10]. Both subsets express relatively high levels of CC chemokine receptor CCR2 and CXCR4. Whereas CCR1, CCR3, CCR4, CCR6, CXCR1, CXCR2, and CXCR5 are very weakly, or not expressed, on both circulating myeloid DC and plasmacytoid DC. Conversely, CCR5 and CXCR3 expression is clearly divergent in the two subsets, being low on blood myeloid DC, but high on plasmacytoid DC [10, 40]. In contrast with the overall similar pattern of chemokine receptor expression, circulating myeloid DC and plasmacytoid DC exhibit a profound difference in their capacity to migrate in response to chemokines (Fig. 16.1) with CXCL12 being the only chemokine active in a classic chemotaxis assay [10] or in transmigration assays across an endothelial cell monolayer [41]. In classical chemotaxis assays, the ligands of CXCR3, namely CXCL9, CXCL10 and CXCL11, are inactive in inducing plasmacytoid DC migration but can promote plasmacytoid DC migration in response to CXCL12 [42, 43]. However, it was shown that CXCR3 ligands are fully competent in inducing plasmacytoid DC adhesion and migration when presented to plasmacytoid DC immobilized on the heparan sulfates present on endothelial cells membrane, a physiological relevant condition [13, 40, 44].

Plasmacytoid DC are normally absent from peripheral tissues and they are believed to migrate constitutively from the blood into lymph nodes through high endothelial venules [45, 46]. This migration is mediated by L-selectin and is increased, by an E-selectin-dependent mechanism, when lymph nodes are exposed to inflammatory conditions [44, 47]. Accordingly, plasmacytoid DC express high levels of CD62 ligand and PSGL1 the counter ligands of P- and E-selectins [40, 45]. Recruitment of plasmacytoid DC to nonlymphoid tissues is observed in some pathological conditions, such as autoimmune diseases (i.e. lupus erythematosus disease, psoriasis and rheumatoid arthritis) [38, 48, 49], allergic diseases (i.e. contact dermatitis and in nasal mucosa polips) [50] and in tumors [51–53]. However, the mechanisms leading to the recruitment of plasmacytoid DC to inflammatory sites remain unresolved. Recently, chemerin, a new chemotactic factor was proposed as a key signal for the recruitment of plasmacytoid DC into pathological tissues (see below) [12].

Under inflammatory conditions an array of chemokines are expressed by resident cells and presented by activated endothelial cells, these include CCL2, CCL5, CCL7, CCL13, CCL20 and CCL22 [26, 54]. DC present at the site of immune reaction also represent an important source of inflammatory chemokines (see below). Pro-inflammatory mediators, such as TNFα and IL-1 produced by tissue macrophages will further contribute to the expression of inducible chemokines by virtually all cells resident in the area. As previously mentioned, immature DC express a repertoire of chemokine receptors that can interact with inflammatory chemokines (Fig. 16.1). At the inflammatory site, endothelial cells upregulate the expression of adhesion molecules, including E- and P-selectin, ICAM-1, VCAM-1 and L-selectin ligands, all of which are involved in leukocyte tethering and rolling [9].

16.3
Migration of Mature Dendritic Cells to Secondary Lymphoid Organs

A dramatic change in the repertoire of chemokine receptors is promoted by DC activation. This change is responsible for the migration of DC from the periphery to the draining lymph nodes. The signals that promote this process include a variety of maturation factors, such as IL-1, TNF and LPS [55–57]. Exogenous administration of these molecules promotes the loss of DC from the periphery within a few hours (i.e. 6–10 h); mice treated with neutralizing antibodies to IL-1 or TNF show an impaired mobilization of DC [58]. Many microbial products, such as the activators of Toll-like pattern recognition receptors (TLRs) and some endogenous molecules, like CD40L, expressed on the surface of activated T cells may also promote DC activation. DC activation is usually associated with the acquisition of a mature phenotype consisting in an upregulation of co-stimulatory and MHC class II molecules. Activation of DC is also associated with downregulation of chemokine inflammatory receptors and the *de novo* expression of CCR7, the receptor for CCL19 and CCL21, two chemokines that are expressed at the luminal side of high endothelial cells and in the T cell rich areas of secondary lymphoid organs, like tonsils, spleen and lymph nodes [56, 59, 60]. Within the T cell area, CCL19 is expressed by mature interdigitating DC, whereas CCL21 is expressed by stromal cells, as indicated by the severe reduction of expression in the lymphotoxin-$\alpha^{-/-}$ stromal cell deficient mice [61].

The regulation of chemokine receptors by both inflammatory and anti-inflammatory stimuli is not a peculiar feature of DC. Studies performed with mononuclear phagocytes and lymphocytes have shown that cell activation is associated with a drastic change in the repertoire of chemokine receptors. In particular, pro-inflammatory agonists (e.g. LPS, IL-1, and TNF) induce a down modulation of CC chemokine receptors in monocytes and neutrophils, while anti-inflammatory signals, such as IL-10, have an opposite effect [62–66]. *In vitro* exposure of DC to LPS, IL-1, and TNF, or the culture in the presence of CD40 ligand, induced a rapid (<1 h) inhibition of chemotactic response to CCL3, CCL4, CCL5, CCL7, C5a and fMLP [55, 56, 67–69]. Receptor desensitization by endogenously produced chemokines is

likely to be responsible for this effect, however, the reported desensitization to C5a and fMLP, two chemotactic factors that are not produced by activated DC, implicates additional agonist-independent mechanisms [55, 68]. As previously observed in phagocytic cells [63, 66], during maturation inhibition of chemotaxis is followed, with a slower kinetics, by the reduction of membrane receptors and by the down regulation of mRNA receptor expression [55, 56]. Concomitantly, the expression of CCR7 and the migration to CCL19 and CCL21 is strongly up regulated, with a maximal effect at 24 h.

The crucial role of CCR7 and its ligands is clearly observed *in vivo* in mice deficient for these proteins. In mice homozygous for an autosomal recessive mutation, Paucity of Lymph Node T cells (plt), naive T cells fail to home to secondary lymphoid organs. The plt mutation is associated with the expression of CCL21 outside of secondary lymphoid organs, and in a defect in the expression of CCL19 [70]. As a consequence of the lack of CCL21 within secondary lymphoid organs, DC from these mice fail to accumulate in the spleen and in the T cell areas of lymph nodes [15]. Similarly, *CCR7*$^{-/-}$ mice showed a defective architecture of secondary lymphoid organs and a defective homing of DC and lymphocytes [16].

CCR7 expression by DC was shown to be required also for the entry of DC into lymphatic vessels at peripheral sites both in steady state and inflammatory conditions [71, 72]. *CCR7*$^{-/-}$ mice are characterized by the absence of CD11c^{+}MHCII^{high} DC, a subpopulation of DC that is postulated to migrate in a semimature state of activation, from the skin to the draining lymph nodes to maintain tolerance under steady-state conditions [71]. During inflammation, the entry of DC into lymphatic vessels is boosted by the upregulation of CCL21 on lymphatic endothelial cells. Therefore, inflammatory stimuli not only promote the recruitment of immature DC into tissues but also initiate their maturation process and boost the recruitment of maturing DC into lymphatics [72].

The migration pathway that leads DC from periphery to secondary lymphoid organs is still poorly understood and may involve multiple signals in addition to CCR7. In a recent study it was proposed that CCR8 and its cognate ligand CCL1 are involved in the emigration of mouse monocyte-derived DC out of the skin. Furthermore, *in vitro*, the reverse transmigration of human monocyte-derived DC was significantly inhibited by the presence of blocking CCR8 antibodies. Since CCL1 is expressed in the subcapsule of the lymph nodes, it is possible that CCL1/CCR8 may function downstream the entry of DC into the lymphatic by regulating the entry of the afferent DC in the subcapsular sinus of the lymph nodes [73]. Mature DC are also known to express chemotactic receptors other than CCR7, although their biological relevance is still unclear. HCR is an orphan chemokine receptor that was shown to be induced during DC maturation [74]. Our ongoing studies have outlined an interesting pattern of regulation of this receptor in DC. Indeed this putative chemokine receptor is rapidly induced in maturing DC with a kinetics that precedes, and apparently does not overlap with, the expression of CCR7 (data not shown). Therefore, HCR might also play a role in the early phase of DC egression from peripheral tissues. Earlier studies have shown that the expression of CXCR4, the CXCL12 receptor, is retained during DC maturation and mature monocyte-de-

rived DC were shown to migrate in response to CXCL12 [68, 69], however, blood DC matured *in vitro* apparently do not express functional CXCR4 [10]. Similarly, mature DC express ChemR23 [19] and FPRL2 [75] the receptors for two nonchemokine chemotactic factors active on immature DC (see below), but both receptors are apparently nonfunctional in mature DC [12, 19, 20].

The relevance of chemotactic receptors in DC traveling *in vivo* has been clearly documented in mice lacking the gamma isoform of phosphoinositide-3 kinase (PI3Kγ) [14]. PI3Kγ is located downstream seven-transmembrane chemotactic receptors and plays a non-redundant role in cell migration in response to chemotactic agonists [76]. DC generated from PI3Kγ null mice show a profound defect in the migration in response to both inflammatory and constitutive chemokines. A defect of DC migration was also observed *in vivo* in $PI3K\gamma^{-/-}$ and most importantly, this defect was associated with a defective ability of $PI3K\gamma^{-/-}$ mice to generate a specific immune response [14]. Therefore, PI3Kγ may represent a new valuable target to control inappropriate activation of specific immune responses.

Migration is a multistep process that involves the adhesion with endothelial cells and the interaction with physical obstacles, such as basement membranes and collagen meshwork. Mice defective in β2 integrin function [77] and α6 integrin [78] showed a reduced ability in the migration of cutaneous DC to the draining lymph nodes. Conversely, DC generated from mice defective for JAM-A, an adhesion molecule expressed at endothelial cell junctions and by leukocytes, showed a selective increase in their capacity to transmigrate across lymphatic endothelial cell *in vitro*. *In vivo*, *Jam-A*$^{-/-}$ mice have an enhanced migration of skin DC to lymph nodes and an increased response in a contact hypersensitivity model [79]. Similarly, *in vivo* DC migration was increased in SPARC null mice [80]. SPARC (Secreted Protein, Acidic and Rich in Cysteine) is a calcium-binding matricellular glycoprotein that binds a range of extracellular matrix components. The facilitated migration of DC in *SPARC*$^{-/-}$ mice was associated to an increased ability to mount an antigen specific immune response [80]. Consistently with the notion that the interaction with extracellular matrix components may represent an obstacle for the migration of DC, matrix metalloproteinase (MMP)-9 and -2 are required for the migration of skin DC *in vivo*. *MMP-9*$^{-/-}$ mice present a severe defect in the egression of Langerhans cells from skin *ex vivo* and *in vivo* [81], and for the recruitment of DC into the airways of antigen-sensitized mice [82]. CD44 is a heterogeneous multifunctional protein that functions as a major cell surface receptor for hyaluronate, an extracellular matrix component. CD44 isoforms are differentially modulated during DC maturation and participate in their migration out of the skin and in the induction of the sensitization phase of contact hypersensitivity response [83].

Overall these findings provide a model for DC trafficking in which activation of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR4, CCR5 and CCR6) and the regulation of adhesion molecules and protease secretion, function as signals to localize immature DC or their precursors to peripheral tissues. After Ag uptake, immune/inflammatory stimuli induce DC maturation and the loss of responsiveness to inducible chemokines locally produced. This unresponsiveness plays a permissive role for DC to leave peripheral tissues. Meanwhile the slower up regu-

lation of CCR7, and possibly other chemotactic receptors, prepare DC to respond to CCL19 and CCL21 expressed in lymphoid organs.

16.4 Chemotactic Factors for Dendritic Cells: more than Chemokines

DC express a wide variety of receptors for chemotactic agonists different from chemokines (Table 16.1). These include receptors for bacterial components, bioactive lipids and for signals of "tissue danger". These chemotactic stimuli are rapidly produced (within minutes) at the site of inflammation and represent an early signal for the recruitment of DC, or their precursors, that can precede chemokines action.

Early work performed by this group documented that myeloid immature DC, but not mature DC, express functional receptors for formylated peptides (fMLP) and chemotactic components of the complement cascade (i.e. C5a) [17]. The expression of C5a receptors was also confirmed *in vivo* in Langerhans cells [84]. The formyl peptide receptor family includes multiple proteins, two of them FPR and FPRL2 were found to be expressed by immature DC [75]. FPR is the fMLP receptor, whereas FPRL2 is activated by the WKYMVm hexapeptide originally identified from a combinatorial peptide library for its ability to stimulate phophoinositide hydrolysis in lymphocytes [85]. Recently, F2L an endogenous high affinity ligand for FPRL2 was isolated from porcine spleen [20]. F2L is a highly conserved acetylated 21-aminoacid peptide derived from the cleavage of the N terminus of the intracellular heme-binding protein HBP that activates FPRL2 in the low nanomolar range. The acetylation of the N-terminal methionine of F2L is a modification that is reminiscent of the formylated methionine of bacterial and mitochondrial peptides active on FPR. F2L is able to induce calcium mobilization and chemotaxis of monocytes and immature DC. Therefore, F2L could be involved in the response of these

Tab. 16.1 Nonchemokine chemotactic receptors expressed by immature dendritic cells.

Chemotactic receptor	*Agonist*
ChemR23	Chemerin
C5aR	C5a
FPR	fMLP
FPRL1	LL-37
FPRL2	F2L
PAFR	PAF
CCR5	HisRS, AsnRS
CCR6	β-defensin
CXCR3, CXCR5	S-antigen, IRBP
?	α-defensin
?	EDN

Histidyl-tRNA synthase (HisRS); asparaginyl-tRNA synthase (AsnRS); Interphotoreceptor Retinoid Binding Protein (IRBP; Eosinophil-Derived Neurotoxin (EDN).

cells to infection, inflammation or cell death. DC are known to process and present antigens derived from infected, malignant or allogeneic cells. Recent evidences indicate that DC are attracted by dead or dying cells [86]. The first self-molecules shown to represent danger signals were the heat shock proteins gp96 or hsp70. These proteins are released from necrotic cells and are able to potently activate antigen-presenting cells [87]. Intracellular nucleotides released under conditions of hypoxia, ischemia, inflammation or mechanical stress, and crystalline uric acid deriving from dead cells, were also shown to stimulate DC [88, 89]. It is conceivable that F2L, released from HBP by proteolysis following cell suffering or cell death, contributes to the chemoattraction of monocytes and DC through FPRL2. F2L and FPRL2 might therefore be involved in the development of a number of inflammatory diseases associated with cell death [20].

Human and mouse DC express functional receptors for platelet activating factor (PAF) [18, 90]. Since PAF is synthesized at the site of inflammation following PLA2 activity, it is conceivable that it functions to recruit DC to the pathological site. Using a FITC skin painting assay, it was recently reported that the migration of Langerhans cells to skin draining lymph nodes is increased in *Pafr*$^{-/-}$ mice and in wild type animal administered with a PAFR antagonist. Similarly, the PAFR antagonist promoted the egression of LC from human skin explants, *in vitro* [90]. These results suggest that PAF normally acts as a negative regulator of DC mobilization from skin to lymph nodes. The retention ability of PAF into peripheral tissues may be relevant in the retention of DC at pathological sites, such as atherosclerotic plaques [90].

One of the effector mechanisms of innate immunity relies on the generation of antimicrobial substances, these include inorganic cytotoxic molecules (i.e. hydrogen peroxide and nitric oxide) and antimicrobial peptides, like defensins and cathelicidins. The latter, in addition to their antimicrobial function, possess chemotactic activity for immature DC. Human α-defensins and β-defensins are chemotactic for immature, but not mature DC [91]. The chemotactic activity of β-defensins is mediated by the interaction with CCR6, the receptor for CCL20. Although the migration in response to α-defensins is inhibited by pertussis toxin, the identity of the receptor for these proteins has not yet been identified [91]. Sharing a receptor with chemotactic factors is not unique to β-defensins, because LL-37, an antimicrobial peptide, promotes the migration of human neutrophils, monocytes and T lymphocytes through the interaction with FPRL1 [92]. Chemerin, a new chemotactic protein that belongs to the cathelicidine family, was shown to induce the migration of both myeloid and plasmacytoid DC through the interaction with ChemR23 [12]. Chemerin is a novel chemotactic protein identified as the natural ligand of ChemR23, a previously orphan G protein-coupled receptor expressed by immature dendritic cells and macrophages [19]. Chemerin was purified from ovarian cancer ascites and found to correspond to the product of the Tig-2 gene. Chemerin is expressed by many tissues, including spleen and lymph nodes, and is secreted as prochemerin, a poorly active precursor protein. Extracellular proteases involved in the coagulation cascade [93] or released by leukocytes convert prochemerin into a full agonist of ChemR23 by proteolytic removal of the last six amino acids [19].

ChemR23 is expressed by blood plasmacytoid DC and chemerin was found active in inducing their transmigration across an endothelial cell monolayer. *In vivo* ChemR23 was expressed by plasmacytoid DC localized in reactive lymph nodes and in skin lesions of lupus erythematosus patients. Of note, chemerin, was selectively expressed by high endothelial venules in lymph nodes and by dermal blood vessels in lupus skin lesions. These results strongly suggest that the ChemR23/chemerin axis is likely to play a key role in regulating the trafficking of plasmacytoid DC to lymph nodes and to pathological tissues [12]. Eosinophil-derived neurotoxin (EDN), a protein belonging to the ribonuclease A superfamily was found to be a selective chemotactic agonist for DC. The effect of EDN is sensitive to the action of pertussis toxin, however, the nature of the chemotactic receptor engaged on DC membrane is still unknown [94].

Recent work has shown that DC may have a pivotal function in the induction of autoimmunity [95]. Histidyl-(HisRS) and asparaginyl-(AsnRS) tRNA synthases, two cytoplasmic proteins involved in protein synthesis that function as autoantigens in myoisitis, were shown to induce the migration of immature DC through the interaction with CCR5 [96]. More recently, S-antigen and interphotoreceptor retinoid binding protein (IRBP) two self-antigens involved in autoimmune uveitis were shown to bind and activate CXCR3 and CXCR5 on immature DC [97]. Therefore, self-antigens may promote autoimmunity also through the recruitment of antigen presenting cells at the site of tissue injury.

16.5 Tuning Dendritic Cell Migration by Nonchemotactic Signals

Multiple evidences have shown that chemokine receptor expression is not predictive of DC migration suggesting that the coupling of chemokine receptors to chemotaxis is also regulated at the signaling level [10, 21]. For instance the simultaneous exposure of DC to maturation factors and anti-inflammatory cytokines (i.e. IL-10) uncouples inflammatory chemokine receptors from chemotaxis and converts them in scavenging chemokine receptors [21]. Recent findings revealed that eicosanoids, such as cysteinyl leukotrienes (cysLTs) and prostaglandin E2 (PGE2) regulate CCR7-dependent migration of DC to lymph nodes (Fig. 16.2) [24, 98]. CysLTs derive from the 5-lipoxigenase (5-LO) pathway of arachidonic acid metabolism. Ex-

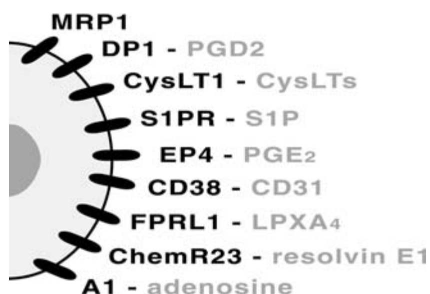

Fig. 16.2 Factors that regulate dendritic cell migration. Several agonists are known to promote (CystLTs, S1P, PGE2, CD31), or inhibit (PGD2, LPXA4 and resolvin E1) the migration of dendritic cells. A1, adenosine receptor A1; CystLTs, cysteinyl leukotrienes; S1P, sphingosine 1-phosphate; PGE2, prostaglandin E2; PGD2, prostaglandin D2; LPXA4, lipoxin A4.

perimental evidence about the role of cysLTs in DC migration emerged by the use of MRP1 blocking antibodies and from studies in mice lacking the lipid transporter multidrug resistance protein 1 (MRP1) [99, 100]. In the absence of MRP1 the migration of epidermal DC to the draining lymph node was impaired and the exogenous administration of LTC4 or LTD4 could rescue the defect. DC express the cysLTs receptor CysLT1, and *in vitro*, cysLTs promoted DC migration in response to the CCR7-ligands CCL19 and CCL21. Therefore, the MPR1-mediated efflux cysLTs and autocrine or paracrine activation of cysLTR promote the migration of maturing DC. A role in the activation of lung DC functions by cysLTs was recently reported in a mouse model of allergy [46, 101].

FTY720 is a synthetic derivative from ISP-1 (myriocin), a compound from traditional Chinese medicine. FTY720 was recently shown to prolong allograft survival by increasing the migration of T lymphocytes to CCR7 ligands and by promoting their sequestration in secondary lymphoid organs [102, 103]. FTY720 is phosphorylated to a phosphate ester (P-FTY720) that is analogous to sphingosine 1-phosphate (S1P) and acts through the S1P receptors [104]. FTY720 was shown to induce the migration of immature DC [105] and to inhibit the production of Th1 cytokines [105, 106].

PGE2 is an arachidonic acid metabolite generated by PGE2 synthases downstream of cyclooxygenases. PGE2 modulates multiple aspects of DC biology, such as maturation, cytokine production, T cell activation and apoptosis [24]. Furthermore, PGE2 promotes the migration of mature human monocyte-derived DC to the CCR7 ligands CCL19 and CCL21 [107, 108]. The effect of PGE2 on these cells is mediated by two of the four PGE2 receptors, namely EP2 and EP4 and the cAMP pathway. Interestingly, blood myeloid ($CD1b/c^+$) DC, matured *in vitro*, did not require PGE2 for an optimal migration in response to CCR7 ligands [108]. These results suggest that the coupling of CCR7 to chemotaxis is regulated by the state of activation/maturation of DC. The importance of PGE2 for DC migration has been highlighted *in vivo* by the use of mice that are genetically defective for EP4 [109]. *Ptger4*$^{-/-}$ mice displayed a reduced migration of skin Langerhans cells to regional lymph nodes after FITC sensitization, *in vivo*, and a reduced spontaneous emigration from skin explants, *ex vivo*. The nonredundant role of EP4 in Langerhans cell migration was further confirmed in wild-type mice by the use of an EP4 antagonist, and correlated with an impaired induction of contact-type hypersensitivity responses [109]. PGE2 is secreted by monocytes, macrophages, fibroblasts activated by inflammatory stimuli [110]. This suggests that immature DC are exposed to PGE2 in situ at the site of inflammation. PGE2 is known to induce or to modulate the migration of a variety of cell types, including endothelial cells, mesangial cells, tumor cells, human monocytes and *Oesophagostomum dentatum* larvae [108, 111]. This suggests a more universal mechanism of action of PGE2 that may go beyond the effect on DC.

PGD2 is a prostaglandin released by mast cells during allergic response that interact with two different receptors, the D prostanoid receptor 1 (DP1) and DP2, also known as CRTH2 [112]. PGD2 was shown to inhibit Langerhans cell migration to the skin draining lymph nodes during percutaneous infection with the hel-

minth parasite *Schistosoma mansoni* [112] and the migration of lung DC *in vivo* [113].

New anti-inflammatory lipid mediators were recently identified during the resolution of inflammation and following aspirin treatment [114]. These compounds were shown to inhibit leukocyte migration by interacting with chemotactic receptors. Among them, lipoxin A4, an autacoid generated from arachidonic acid, was found to inhibit DC mobilization in the spleen through the interaction with FPRL1 [115]. Similarly, resolvin E1, a new bioactive oxygenated product of eicosapentaenoic acid, a component of fish oils, reduced dermal inflammation, IL-12 production and DC migration through the interaction with ChemR23, the chemerin receptor [116]. Therefore, the engagement of the same receptor by alternative ligands may result in an opposite biological output.

CD38 is an ectoenzyme that catalyses the synthesis of cyclic ADP ribose (a potent second messenger for calcium release), as well as a receptor that initiates transmembrane signaling upon engagement with its counter-receptor CD31. CD38 was found to be up regulated during DC maturation and to promote a DC membrane and functional phenotype *in vitro* [117]. CD38 apparently controls DC migration, since mice lacking CD38 have a defect in the recruitment of DC precursors to peripheral tissues and in DC mobilization of mature DC to the draining lymph nodes [22].

16.6 Concluding Remarks

Dendritic cells (DC) are professional antigen presenting cells. To accomplish their biological function they need to go through a complex pattern of migration which includes their localization to both peripheral nonlymphoid tissues and secondary lymphoid organs. In the absence of correct tissue localization, DC fail to promote proper immune responses. DC differentiate along multiple pathways and are characterized by different states of maturation, each one associated with peculiar functions and membrane phenotypes. The first description of chemotactic factors active on DC was published by this group ten years ago. However, there is a considerable array of nonchemokine chemotactic signals and of molecules that modulate DC migration; for some of them a complete understanding of the mechanism of action is still missing. A better understanding of the signals involved in the migration of DC subsets *in vivo* constitutes a valuable basis for the development of new strategies for the control of DC migration and function under pathological conditions.

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VI
T-cell Activation and Co-stimulation

17
Antigen Processing and Presentation: CD1d and NKT cells

Serani L.H. van Dommelen, Dale I. Godfrey and Mark J. Smyth

17.1
Introduction

Dendritic cells (DC) function as the professional antigen-presenting cells (APC) of the immune system, conventionally presenting protein antigens using MHC class I and class II molecules. In contrast to conventional T lymphocytes, whose T-cell receptor (TCR) recognizes peptide antigens in the context of classical MHC class I- or II-presentation, CD1d-restricted NKT cells express a TCR that recognizes a glycolipid antigen presented by a nonclassical antigen-presenting molecule, CD1d [1, 2]. Specifically, NKT cells express a semi-invariant TCR, indicating that they recognize comparatively conserved structures, rather than the diverse set of antigens/presenting molecules recognized by conventional T cells. This distinct immune-recognition system and other NK cell receptors expressed by NKT cells define these as T cells that bridge the gap between the innate and adaptive immune system. NKT cells rapidly release a variety of cytokines following TCR ligation allowing them to regulate both innate and adaptive immune responses in the context of antitumor immunity and antimicrobial immunity, and the balance between tolerance and autoimmunity.

17.2
CD1d and Antigen Presentation

17.2.1
The CD1d Molecule

The CD1 antigen-presenting molecules are evolutionarily conserved [3]. Five CD1 genes have been found in humans, encoding proteins that segregate, based on se-

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quence homologies, into group 1, containing CD1 a, b, c, group 2, containing CD1d and an intermediate group containing CD1e [3, 4]. Mice only contain two genes expressing CD1d proteins, CD1d1 and CD1d2, which are highly homologous [5]. The CD1 molecules are all antigen-presenting molecules that present monomeric glycolipids to the immune system, however, for the purposes of this chapter, the CD1d molecule is discussed in detail since it restricts NKT cells.

Glycolipid-binding CD1d proteins are type 1 integral membrane proteins and are nonpolymorphic major histocompatibility complex (MHC) class 1-like glycoproteins. Like the MHC class I molecule, CD1 proteins consist of $\alpha1$, $\alpha2$, and $\alpha3$ domains and are anchored to the cell membrane by a transmembrane domain followed by a short cytosolic tail [6]. Although there is little amino acid sequence homology between CD1 and classical MHC class I molecules, the crystal structure of

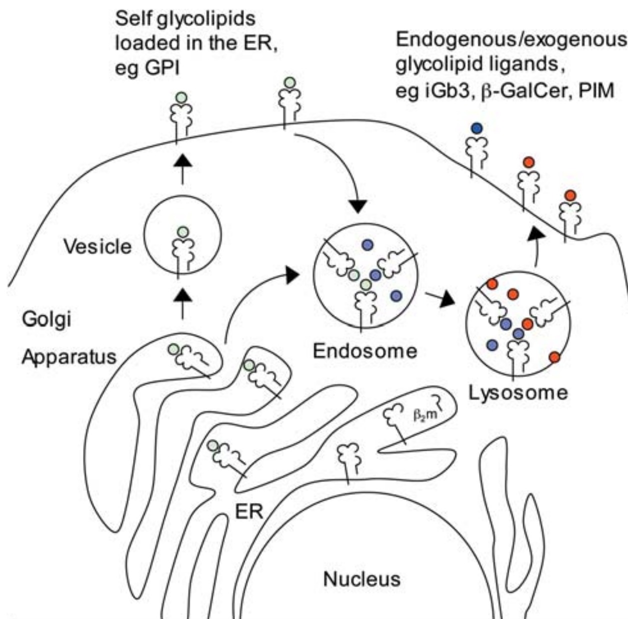


Fig. 17.1 Intracellular trafficking pathway of newly synthesized CD1d molecules. The CD1d heavy chain is folded and assembled in the endoplasmic reticulum (ER), and associates with a β_2m molecule. A self-glycolipid (indicated in green), such as GPI, binds to the CD1d molecule in the ER. The glycolipid loaded CD1d molecule is processed along the secretory pathway to the plasma membrane. A proportion of CD1d molecules associate with MHC class II/Ii chain complexes and are directed to endosomal compartments. CD1d molecules located on the plasma membrane are internalized, traffic through the late endosome/lysosome compartments, due to the internalization sequence within the cytoplasmic tail of CD1d, and are recycled back to the plasma membrane. In the endosome/lysosome compartment, CD1d exchanges the lipid it bound in the ER for a foreign glycolipid (indicated in purple) such as PIM from bacteria, encountered as free soluble molecules in the endosome, or another self-glycolipid, potentially the cellular derived iGb3 (indicated in red), encountered in the lysosome as a degradation product.

the mouse CD1d protein showed that its three-dimensional structure is mostly superimposable with MHC class I [7]. Again, like MHC, CD1 proteins are expressed as heterodimers containing the glycosylated form of the CD1 heavy chain paired with β2-microglobulin [8, 9].

Unlike the MHC molecule, CD1 proteins are nonpolymorphic and present glycolipids rather than peptides to T cells [3]. Therefore, in accordance with their differences in antigen presentation, the groove of the CD1d molecule varies from that of the MHC molecule as it is deeper and shaped into two pockets that are lined by nonpolar residues to mediate glycolipid antigen presentation. Glycolipid antigens bind to the CD1 molecule using hydrophobic interactions by inserting their hydrophobic tail into the groove of CD1, allowing the exposed polar head group of the glycolipid to make contact with the TCR [10]. Indeed, the crystal structure of mouse CD1d shows a very hydrophobic binding groove that is closed at both ends and contains two deep, large pockets (A' and F'). The head group of the glycolipid is exposed via the top of the molecule through a small opening located at the centre of the groove and extending to a point over the centre of the F' pocket [7]. The structure of the CD1d groove thus allows the structurally diverse component of the glycolipids to be presented for recognition by TCR, whereas the alkyl chains of glycolipids are comparatively structurally homogeneous.

17.2.2 Nature of CD1d and Glycolipid Recognition by TCR

The biophysical characteristics of the antigen/antigen-presenting molecule interactions by CD1 molecule presentation of glycolipid antigens to TCR are different to conventional peptide antigen presentation by MHC to TCRs. For example, CD1d/TCR interacts with a higher affinity than the conventional MHC/TCR interaction, due to much slower off rates [11, 12]. Unlike the interaction between MHC and conventional αβ TCRs, the NKT-cell TCR and CD1d/α-GalCer interaction is based on an inflexible interface involving the more rigid structure of the glycolipid sugar moiety and the invariant Vα14 chain of the TCR on the NKT cell [12, 13].

However, successful presentation of a glycolipid as an antigen must fulfill the following requirements; the glycolipid must bind efficiently to the antigen-presenting molecule CD1d, the antigen-presenting molecule and glycolipid complex must be sustained for a sufficient period of time to interact with the TCR of the CD1d-restricted T cells, and finally, the glycolipid must be efficiently loaded into the antigen-presenting molecule. To address the first requirement, glycolipids bind efficiently to CD1d due to the structural characteristics of CD1d, however, a low specificity for CD1d binding to glycolipids is inherent in this monomorphic, hydrophobic type of interaction. To address the second requirement, it has been shown that *in vitro* the half-life of the CD1d-glycolipid complex is 24 h or longer [12, 14], allowing efficient interaction between glycolipids and TCR molecules. Possibly, it is through the third requirement, the successful loading of glycolipid into the CD1d molecule that the various synthetic and cellular glycolipids are specifically presented by CD1d [15–17]. The CD1 molecules proceed along an intracellular trafficking

route that is different from their MHC counterparts that sample both cytolytic and endosomal antigens (Fig. 17.1). Generally, CD1d-mediated presentation of a glycolipid antigen to NKT cells requires trafficking of the CD1d molecule to the late endosome and lysosome [15, 16, 18], but not always [19]. Initially, newly synthesized mouse CD1d molecules may assemble with β2-microglobulin in the endoplasmic reticulum (ER), bind self-lipids such as GPI, and subsequently traffic to the plasma membrane along the secretory route [20]. A proportion of CD1d molecules associate with MHC class II/Ii chain complexes and are directed to endosomal compartments. Plasma membrane located CD1d molecules are internalized and traffic through the early and late endosomal compartments to the lysosomes by adaptor–protein complexes (AP3) [21, 22] that recognize a tyrosine motif in the cytoplasmic tail of CD1d [15, 20, 23, 24]. Glycolipid antigens are then loaded into the CD1d molecule in the late endosome/lysosome, a step that has proven to be absolutely required for antigenic glycolipid presentation as CD1d molecules truncated in the cytoplasmic tail and, hence, defective for passage through lysosomes, do not stimulate NKT cells [15, 16]. Lipid transfer proteins (LTP), such as the saposins and GM2 activator proteins, colocalize with CD1d in the late endosomal/lysosomal compartments [25, 26]. LTPs have been shown to be necessary, in an *in vitro* model, to extract glycolipids that are embedded in membranes and interacting with CD1d, based on affinity with their polar heads. Due to these properties, LTPs are assigned the functions of lipid loading, as well as lipid editing [27]. In addition, endosomal enzymes are involved in regulating antigen processing and presentation by CD1d. For instance, it was shown that the lysosomal enzyme α-galactosidase A was involved in the processing of a disaccharide analog of α-GalCer prior to its presentation by CD1d, suggesting that complex glycolipids can be processed to antigenic monosaccharides [19]. In addition, the lysosomal cysteine protease cathepsin S (catS), is required for the cleavage of the chaperone molecule invariant Ii chain [28]. Mice deficient for catS were shown to be impaired in NKT-cell selection and function, thought to be due to an alteration in trafficking of CD1d in DC, where CD1d accumulated in the endosome colocalized with the Ii chain, resulting in a reduced ability to present antigen to NKT cells [29]. However, a subsequent study refuted this result, showing that catS deficient mice had normal NKT-cell development, while in that study, catL was found to be important in NKT-cell development and CD1d antigen presentation [30]. The basis for the different results with CatS deficient mice is unclear but may be due to the different staining methods used in these studies to detect NKT cells. CatL appears to be required for the presentation of self-ligands by CD1d-expressing thymocytes [30].

17.2.3
Nature of CD1d-expressing APC

In humans, CD1d is expressed on cell types of the lymphoid system including most monocytes, immature DCs, mature DCs, and macrophages [31]. CD1d expression has also been detected on dermal DCs, but not on Langerhans cells [32]. Substantial levels of CD1d expression are found on circulating B cells, splenic B

cells and mantle zone B cells in the lymph node [33]. Although CD1d is not detected on resting mature T cells in humans [33], it is expressed when these cells are activated with phytohemagglutinin (PHA) [33]. CD1d is also expressed on human cortical thymocytes [33], which may be important for NKT-cell selection, as it is in mice [34]. In human, CD1d is also expressed in nonlymphoid tissue of the gut and liver, epithelial cells, parenchymal cells, and vascular smooth muscle cells [35, 36].

Cell types of the mouse hematopoietic system that express CD1d include professional APCs such as DCs, macrophages and B cells [37–39]. Splenic marginal zone B cells have very high levels of CD1d expression [38], as do a subset of follicular B cells [40]. The expression of CD1d by splenic marginal zone B cells has been implicated in the NKT-cell-mediated induction of T regulatory cells that are responsible for mediating systemic tolerance associated with anterior chamber-associated immune deviation (ACAID) [41]. In addition, CD1d is expressed on immature and mature thymocytes and on peripheral T cells [37–39]. CD1d-expressing thymocytes [34], and not thymic epithelial cells [42], are required for the positive selection of CD1d-reactive T cells. However, thymic stromal cells are required to regulate the maturation/expansion of positively selected NKT precursors, involving the nuclear factor (NF)κB–inducing kinase (NIK)/NFκB2/RelB pathway [43, 44]. Mouse CD1d is also expressed on nonhematopoietic cells including hepatocytes and, albeit controversially, on the gastrointestinal epithelium [45]. Two studies failed to detect CD1d expression on intestinal epithelial cells by immunohistochemistry [37, 39]. In mice, the CD1d2 protein is expressed by thymocytes, but apparently not by peripheral lymphocytes, and it is not involved in the development of CD1d-restricted T cells [46, 47].

The physiological relevance of all the different CD1d-expressing cell types as functional APC is not yet known. There is substantial evidence to show that DC successfully present α-GalCer in the context of CD1d to the TCR of CD1d-restricted T cells, as will be discussed further in this chapter. CD1d expression on DC has been carefully evaluated, and it has been shown that mature myeloid DC derived from human peripheral blood monocytes express the CD1d molecule on the cell surface [48]. Furthermore, immunohistochemical analysis showed that CD1d expression *in vivo* is preferentially located in the T-cell-dependent lymphoid regions of lymph nodes, as it is expressed on DC located in the paracortical T-cell zones of lymph nodes, implying that this may be a physiological site of interaction for DC and human NKT cells [48]. A study examining the different the gene expression patterns in mouse splenic $CD11c^{high}$ DC subsets using microarray and flow cytometric analyses demonstrated that, while the CD1d molecule was expressed on all DC subsets, it was expressed at higher levels by the $CD8\alpha^+$ DC subset (lymphoid-related DC) in comparison to the $CD8\alpha^-CD4^-$ (DN) and $CD4^+$ DC subsets [49]. The physiological importance of DC subsets in the activation of NKT cells is not yet known, but may involve regulation of the NKT-cell response towards a Th1 or Th2 phenotype. In support of this, a study has demonstrated that the *in vitro* interaction of immature neonatal human NKT cells with matured monocyte-derived DC induced polarization towards IFN-γ production, and this outcome was CD1d-dependent [50]. Conversely the interaction with DC matured from $CD4^+CD11c^-$ plasma-

cytoid cells polarized neonatal NKT cells towards IL-4 production, and this outcome was not dependent upon CD1d [50].

17.3
CD1d-restricted NKT Cells

17.3.1
Defining NKT Cells

CD1d-restricted T cells are immune effector cells that recognize a glycolipid antigen presented only in the context of CD1d. These cells are by definition T cells as they express a TCR. In both humans and mice, many CD1d-restricted T cells also co-express, NK1.1 (NKR-P1 in humans and CD161c in mice), a marker characteristic of NK cells, and based on this, have been loosely defined as natural killer T (NKT) cells (reviewed in Refs. [2, 51]). Other NK-cell specific markers found on NKT cells include CD56, CD94, and some mouse NKT cells express the inhibitory NK receptors Ly49A, Ly49C/I and Ly49G2 [51–54]. Human NKT cells are 50% positive for expression of CD94, a C-type lectin [55]. Human NKT cells appear to be negative for the immunoglobulin superfamily killer cell Ig-like receptors (KIRs) CD158α/β [56]. NKT cells express markers shared by both NK cells and activated T cells, including CD5, CD44, CD69, the IL-2 receptor β chain (CD122), Ly6C and IL-7 receptor α chain (IL-7Rα, CD127) [57–59]. However, it is necessary to elaborate on the definitions of NKT and CD1d-restricted T cells since both terms encompass a variety of cellular subpopulations. The term NKT is sometimes used as a phenotypic definition for a complex variety of cells, whereas the term CD1d-restricted T cells is a functional definition, but also encompasses more than one cell type. However, usually the term “NKT” refers to CD1d-dependent T cells, and at least two classes of these cells exist, depending on their TCR expression [2]. Other T cells that express NK receptors also exist, but these represent an extremely diverse population of cells that have little functional overlap with CD1d-dependent NKT cells [2]. The most defined and studied cellular subpopulation is the CD1d-restricted NKT cells, which express an invariant TCR α-chain. In mice, most NKT cells express an invariant Vα14-Jα18 TCR α-chain rearrangement with a conserved CDR3 region, and they are preferentially paired with Vβ8.2, Vβ2, or Vβ7. Similarly, human NKT cells express the homologous Vα24-Jα18 TCR α-chain with a Vβ11-containing β-chain [60–62]. The major discriminating feature of this prototypical NKT-cell subset is their ability to react to the synthetic glycolipid α-GalCer in the context of CD1d-presentation. Indeed, CD1d-restricted NKT cells recognize α-GalCer and both mouse and human NKT cells can be selectively identified by the use of α-GalCer-loaded CD1d tetramers [55–56, 63–66]. However, calling these cells $NK1.1^+$ T cells is not entirely accurate as not all CD1d-restricted T cells that bind α-GalCer/CD1d tetramer express NK1.1 [2]. A second subpopulation of NKT cells is the CD1d-dependent T cells that possess a more diverse, yet still apparently limited, TCR repertoire, including cells that express Vα3.2-Jα9 or Vα8 paired with Vβ8 in

mice [67]. Similar cells have also been identified in humans [68]. In addition, many phenotypic NKT-like cell subpopulations express a diverse TCR repertoire and do not recognize CD1d molecules but instead recognize restriction elements such as MHC class I and II, TL, Qa-1 and H2-M3 [1, 2, 69, 70]. Such cells can include recently activated classical MHC class I-restricted $CD8^+$ T cells and some $\gamma\delta^+$ T cells [71–73].

Therefore, identifying the prototypic NKT cells based solely on a few phenotypic markers does not reflect their reactivity to α-GalCer presented in the context of CD1d. For example, in humans, a large set of T cells express CD161 (5–10% of T cells from peripheral blood mononuclear cells), however, less than 0.1% of the $CD161^+$ cells stain with α-GalCer/CD1d tetramers [56, 63] [74]. In contrast, in mice, the majority of NK1.1 expressing T cells are CD1d-restricted NKT cells, although the exact proportions are tissue dependent [65, 66].

Thus, in this chapter, describing CD1d-mediated glycolipid antigen presentation to NKT cells, the focus will be on the subpopulation of cells that is CD1d-restricted and express the invariant Vα14-Jα18 (mice) or Vα24-Jα18 (humans) TCR rearrangement. These cells have been designated in previous studies as Vα14 invariant (Vα14i) NKT cells, invariant NKT cells (iNKT cells), classical NKT cells, or type I NKT cells (reviewed in refs. [1, 2, 75]), but here will be simply referred to as NKT cells.

17.3.2
Tissue Location and NKT Cell Subsets

In mice, NKT cells appear to preferentially accumulate in liver (10–40% of liver lymphocytes) and are present in the thymus, bone marrow and spleen. NKT cells are rare in lymph nodes [76, 77]. In humans, NKT cells account for approximately 0.2% of peripheral blood T cells and they are clearly less frequent (<1%) in human liver than compared with mouse liver [78–80]. In addition, human NKT cells have been identified and characterized in the bone marrow [81], the gut [82], cord blood [83] and thymus [84, 85].

In mice, NKT cells are predominantly $CD4^+$ or double negative (DN). The composition of CD1d-restricted T cells in mice breaks down as follows; 60–90% are $CD4^+$, 10–40% are DN and very few if any express CD8α or CD8β [65, 66, 77]. In humans, an average of half of the NKT cells express CD4, and up to 50% of cells express CD8α [55] while expression of CD8β is rare [68, 86]. The $CD4^+$ and $CD4^-$ subsets of NKT cells exist in different proportions in a tissue-specific manner [58, 76, 77].

17.4
Nature of the Antigens Presented by CD1d-expressing APC to NKT Cells

The semi-invariant nature of the NKT-cell TCR suggests that NKT cells recognize a more limited range of antigens, in comparison to conventional T cells that recognize a wide range of antigens through a diverse TCR. The identity of some antigen-

ic glycolipid/s loaded into the CD1d molecule in the endosome/lysosome compartments and presented to NKT cells has been revealed, but this remains an area of intense investigation.

17.4.1 Self-ligands

NKT cells appear to recognize endogenous antigens, as NKT-cell hybridomas can be modestly stimulated by exposure to CD1d-expressing APC in the absence of exogenously added antigens [15, 52, 87]. An experimental approach, using mouse CD1d-restricted T-cell hybridomas that recognize recombinant CD1d loaded with glycolipid antigens, has shown that endogenous lipids recognized by NKT cells include the ubiquitous phospholipids, such as phosphatidylinositol (PI), phosphatidylethanolamine, and phophatidylglycerol [88, 89]. Similarly, elution of PI and glycosylphosphatidylinositol (GPI) from CD1d molecules shows that these lipids are normally loaded into these molecules [17, 90]. However, to date, NKT cells have not been demonstrably activated by these self-lipids. In addition, β-anomeric glycosphingolipids are found in mammals [91]. The finding that a synthetic β-GalCer can be recognized by NKT cells and induce their effector function, although at a reduced efficiency than α-GalCer [92], indicates that endogenous β-anomeric glycosphingolipids could function as a physiological ligand for NKT cells. Recently, Zhou and co-workers have identified the glycosphingolipid, isoglobotrihexosylceramide (iGb3), as a potential endogenous NKT-cell glycolipid antigen that is recognized by both human and mouse NKT cells [93]. They also provide evidence that iGb3 may be a key endogenous antigen required for efficient intrathymic NKT-cell development. It yet remains to be ascertained that iGb3 is present in the periphery and thymus of both humans and mice, but nonetheless, this potentially represents a major step forward in the field of NKT-cell biology [94].

Self-glycolipids have a role initially in NKT-cell development, where NKT cells originate from the $CD4^{+}CD8^{+}$ double positive (DP) thymocytes that also give rise to MHC class I- and II-restricted T cells [95]. CD1d-expressing $CD4^{+}CD8^{+}$ DP immature cortical thymocytes presenting an endogenous ligand are responsible for the positive selection of NKT cells [34, 42, 96, 97]. In mice, most CD1d-restricted T cells are defined as $CD44^{hi}$ $CD69^{int}$ $CD62L^{lo}$, while their human counterparts are defined as $CD45RO^{+}$ $CD45RA^{-}$ $CD62L^{-}$ $CD69^{+/-}$ [51, 83, 98] and at least the $CD4^{+}$ fraction expresses CD25 [56]. If these phenotypes are due to antigen-driven stimulation of NKT cells, they do not appear to require exposure to external antigens, but rather self-antigens, since an activated/memory surface phenotype of NKT cells is also found in germ-free mice and in human cord blood [83, 99, 100].

17.4.2 Naturally-occurring Exogenous Ligands

NKT cells may also respond to exogenously derived CD1d-restricted antigens in the context of promoting immune responses. Possible naturally-occurring ligands

include pathogen-derived glycolipids or phospholipids, or other self-antigens derived from glycolipids that are induced in infected cells in response to stress. There is experimental evidence for the specific recognition by NKT cells of a range of pathogen-derived glycolipids presented in the context of CD1d. For example, the mycobacteria-derived phospholipid, phosphatidylinositol mannoside (PIM), and bacterium derived antigens such as the Sphingomonas glycosphingolipids and sulfatide variants, were able to activate human and mouse NKT cells via CD1d [101, 102]. However, there have been conflicting reports regarding mouse NKT cell recognition of GPI-anchored glycoproteins from *Plasmodium* or *Trypanosoma* spp. *in vitro* or how they affect the IgG response to GPI-linked proteins *in vivo* [103–107]. It is known that the other three human CD1 molecules, CD1a, CD1b and CD1c, are able to present foreign microbial lipid and lipopeptide antigens, including several mycobacterial cell wall lipids [10, 108–110] (reviewed in Ref. [70]), and are able to present several other autologous antigens, including gangliosides and some phospholipids. Thus, in line with these observations, it seems reasonable that CD1d molecules are also able to present foreign lipid antigens.

17.4.3 Synthetic Ligands

Until recently, most of the studies on NKT-cell function were determined using a synthetic analog of α-galactosylceramide (α-GalCer), called KRN7000 (reviewed in [111]). α-GalCer was originally isolated from marine sponges, and is a very potent NKT-cell agonist [112]. α-GalCer binds to CD1d and activates both mouse and human NKT cells *in vitro* and *in vivo* [112, 113]. In addition, currently many structurally distinct synthetic glycosylceramides are being investigated for therapeutic purposes. These include α-GalCer analogs, produced by systematic alteration in the length and extent of unsaturation of their *N*-acyl substituents such as OCH [114], synthetic forms of β-GalCer [92, 115] and an α-C-glycoside analog of α-GalCer, α-C-galactosylceramide [116]. Given that α-GalCer (KRN7000) induces a broad range of effector functions, it may be beneficial to identify therapeutic compounds with a more selective activation of NKT cells, thereby tailoring subsequent immune responses to target specific disease conditions.

17.5 Effector Functions of NKT Cells

NKT cells are able to rapidly respond to antigenic stimulation, where their primary function appears to be one of Th1 and Th2 cytokine release. The NKT cell–APC interaction then indirectly regulates the activation of a number of immune effector cells via cytokines and/or cell-to-cell contacts. This mechanism allows the NKT cells to affect both the innate and adaptive immune responses.

17.5.1
Cytokine Secretion and Cytotoxicity of NKT Cells

A characteristic of NKT cells that distinguishes these T cells from naïve MHC class I- and MHC class II-restricted T cells is their ability to secrete cytokines within minutes of TCR stimulation. Following *in vitro* stimulation, both mouse and human NKT cells secrete large amounts of Th1 and Th2 cytokines including IFN-γ, IL-4, IL-2, IL-5, IL-10, IL-13, tumor necrosis factor (TNF) and LT (reviewed in [117, 118]). NKT cells also release other pro-inflammatory factors including granulocyte colony-stimulating factor (GM-CSF), lymphotactin, macrophage inflammatory protein (MIP)-1α and MIP-1β [52, 119]. *In vivo* activation of NKT cells by crosslinking of TCR or mediated by α-GalCer results in the rapid secretion of high levels of IL-4 and IFN-γ (reviewed in Refs. [51, 120]). The rapid production and secretion of cytokines is not due to the presence of preformed IL-4 and IFN-γ proteins in resting NKT cells [65], but is likely to be facilitated by preformed mRNA transcripts for IL-4 and IFN-γ in these cells [121, 122]. Upon TCR-mediated activation NKT cells also develop cytolytic effector mechanisms [123]. NKT cells release perforin and granzymes and express membrane bound members of the TNF family (FasL or TNF-related apoptosis-inducing ligand (TRAIL) [124–127]. The cytotoxicity of NKT cells is enhanced by IL-2 and IL-12 and may be important in some antimicrobial and antitumor immune responses [123, 128, 129], but the significance of this feature of NKT cells remains unclear.

17.5.2
The Initial Cross-talk Between CD1d-expressing APC and NKT Cells

Investigating the role of α-GalCer-activated NKT cells in tumor immune surveillance has helped elucidate the mechanism of cross-talk between NKT cells and the CD1d-expressing APC. As mentioned earlier, there are many different CD1d-expressing cell types that could potentially function as APC, however, their relevant importance in presenting antigens to NKT cells is unknown. Experimental evidence to date points to DC as likely candidates for this function. It has been shown that DC expressing the CD1d molecule, and loaded with α-GalCer, mediate the rapid activation of NKT cells [130–133]. The early interaction between NKT cells and DC involves TCR ligation by the CD1d/α-GalCer complex [134, 135]. In mice, the requirement for additional interactions between co-stimulatory molecules is controversial. A study has shown that NKT cells constitutively expressed CD28 that interacted with CD80 and CD86 located on APC, resulting in enhanced IFN-γ and IL-4 secretion by the NKT cells after TCR-mediated activation [136]. However, another study found that CD28 was not required for the initial cytokine secretion by activated NKT cells [121]. CD40L is subsequently upregulated on α-GalCer activated NKT cells. This allows NKT cells to modulate the activities of DC through CD40/CD40L interaction and IFN-γ stimulation, resulting in DC activation and secretion of IL-12 [130, 137, 138]. In addition, expression of the IL-12 receptor (IL-12R), which is constitutively highly expressed on NKT cells, is upregulated in an

IL-12- and IFN-γ-dependent manner [130, 139]. Expression of the IL-12R on NKT cells and CD40 expression on DC was not involved in the initial production of IL-4 and IFN-γ by NKT cells following α-GalCer stimulation [121]. Available IL-12 and IFN-γ can amplify the activation of NKT cells and augment their IFN-γ secretion [130, 136, 137, 140]. This IFN-γ, in combination with DC-released IL-12, results in the indirect activation of other cells of the immune system. NKT cells express inhibitory NK receptors, such as Ly49A, C/I, and Ly49G2, which appear to modulate the threshold of antigen–receptor signaling [141–143].

Recent studies have further delineated that it is the $CD8\alpha^+$ DC subset that releases IL-12 *in vivo* upon α-GalCer stimulation [144, 145]. Splenic DC are the key APC to activate NKT cells *in vivo* in the mouse spleen [144]. This study also highlighted the possibility that NKT cells may be stimulated in an organ-dependent fashion, since Kupffer cells resident in the liver are the primary APC activating hepatic NKT cells. Even though it appears that different types of APC may be involved in the initial presentation of antigen to NKT cells, it is the DC that play a major role in coordinating the downstream Th1 cytokine response stimulated by α-GalCer, and more specifically the $CD8\alpha^+$ subset, due to their release of IL-12 [144].

17.5.3
Functional Diversity of NKT Cell Responses

Autoreactive NKT cells have been reported to mediate immunological functions in a range of diseases, such as type I diabetes and antitumor immunity, through the secretion of either predominantly Th2 or Th1 cytokines. The mechanism(s) responsible for controlling the Th1/Th2 balance of the cytokines secreted by NKT cells are not well defined. However, experimental evidence exists to support several possible mechanisms by which activated NKT cells may achieve this endpoint.

The immediate secretion (within 2 h) of cytokines by NKT cells following TCR activation by CD1d/α-GalCer recognition is, however, of a Th0-like phenotype as both IFN-γ and IL-4 are secreted and this response is not easily influenced [65, 121, 122]. From that point it appears that Th1/Th2 polarization of cytokine secretion can occur. For instance, α-GalCer activated NKT cells undergo a period of expansion (2–3 days) followed by a contraction phase to regain their homeostatic equilibrium in cell numbers. During this expansion phase, NKT cells were shown to produce Th1-biased cytokines [146]. Conversely, it has been demonstrated that multiple treatments of α-GalCer results in the chronic production of the Th2 cytokine, IL-4 [147–150] although the cellular source of this IL-4 was not determined.

It is possible that the antigen presented by the CD1d molecule influences the cytokine profile secreted by activated NKT cells. In support of this, it has been shown that OCH, an analog of α-GalCer, induced predominantly IL-4 secretion [114, 151], a function that was attributed to the truncated sphingosine of OCH and related compounds [152]. Conversely, α-C-GalCer, a synthetic C-glycoside analog of α-GalCer, acts to stimulate an enhanced Th1-type response in mice [116]. It is unclear whether the cytokine profile produced by NKT cells is as a direct response to the type of TCR stimulation, or if it is due to a different impact that these cells may have on cytokine secretion by downstream cells.

The functional outcome of NKT-cell activation could be due to the activation of distinct NKT subsets that reside in different tissues, or due to the differential recruitment of NKT-cell subsets to sites of inflammation [58, 76, 77, 153, 154]. Evidence exists to show that $CD4^+$ and $CD4^-$ NKT-cell subsets exhibit functional diversity that may be physiologically significant. For example, in NKT cells located in the human peripheral blood, the $CD4^+$ subset selectively produced IL-4, IL-13, GM-CSF and IL-2 as well as IFN-γ and TNF-α. The $CD4^-$ NKT subset selectively produced a Th1 cytokine profile (IFN-γ and TNF-α). The expression of perforin, NK-cell receptors such as NKG2D, CD95L and chemokine receptors also varied between these two subsets [55, 56].

Furthermore, the self-reactivity of NKT cells could also influence their effector functions. Weak responsiveness of NKT cells to self-ligands can be amplified by IL-12 secreted by DC in response to activation of Toll-like receptors (TLR) by microbial products [155]. NKT cells receiving these two activating signals (IL-12 from DC and signaling from self-ligands through CD1d presentation) are then capable of producing large quantities of IFN-γ, but not IL-4 [155].

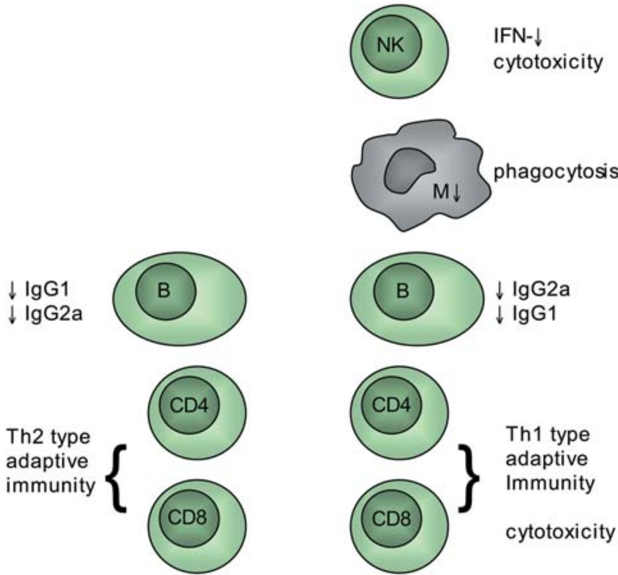
It is possible that the Th1/Th2 phenotype of the adaptive immune response is influenced by interactions between NKT cells and DC. The complex interactions that occur between NKT cells and immature CD1d-expressing DC could result in maturation of DC with different effector phenotypes (i.e. IL-12-producing DC versus IL-10-producing DC). The strength of the TCR signal is thought to define the phenotype of the DC interacting with NKT cells. Thus, a potent TCR signal, such as that provided by α-GalCer, will upregulate CD40L on NKT cells, which, in the presence of IFN-γ, will induce the maturation of DC able to secrete IL-12 [130, 138]. In contrast, the presentation of self-ligands to autoreactive NKT cells delivers a weak TCR signal that does not induce the upregulation of CD40L. In situations where this is combined with the presence of large quantities of IL-4, the maturation of IL-10-secreting DC is foremost [138]. Importantly, a weak TCR signal could be amplified by a cytokine signal (e.g. IL-12), thus eliciting Th1-biased immune responses. Thus, the potency of the antigen presented by CD1d can influence the subsequent response, firstly, the level of NKT-cell activation and secondly, the phenotype of the mature DC. Both will have consequences in the downstream activation of the immune response.

The context in which α-GalCer is presented also influences the Th1/Th2 phenotype of cytokines produced. The response elicited following injection of soluble α-GalCer was distinct from that of bone marrow-derived DC pulsed with α-GalCer. The latter method of antigen presentation resulted in a more prolonged and enhanced production of IFN-γ [156]. Therefore, when α-GalCer is selectively targeted to DCs, the NKT cell-mediated Th1 responses are stronger. α-GalCer has also undergone phase I clinical trial as a soluble agent or when loaded on human peripheral blood DC. A dose escalation study of KRN7000 in advanced cancer patients was first reported [157]. Dose-limiting toxicity was not observed over a wide range of doses and pharmacokinetics was linear in the dose range tested. Immunological monitoring suggested that NKT cells typically disappeared from the blood within 24 h of α-GalCer injection, just as observed in mice. The numbers of

NKT cells are significantly lower in cancer patients compared with healthy controls, supporting previous studies [123, 158]. No clinical responses were recorded and seven patients experienced stable disease. Alternatively, it has been well established that α-GalCer-pulsed DC potently activate and expand human NKT cells *in vitro* [123, 158–162]. Given that similarly pulsed mouse DC can eradicate tumor metastases, a DC-based clinical trial has also been performed where α-GalCer pulsed DC were administered to subjects with metastatic disease [163, 164]. Treatment was well tolerated with no serious adverse reactions and following administration of α-GalCer-pulsed DC, a transient decrease in NKT-cell numbers was observed but numbers subsequently increased. Activation of T cells and NK cells and increased NK cell cytotoxicity was also observed, suggesting the administration of α-GalCer-pulsed DC can activate human NKT cells and subsequently human NK and T cells. Potentially beneficial tumor responses occurred in people with nonbulky disease treated with low to intermediate doses. Future studies may now include prolonged treatment with more mature DC and perhaps a more focused diseased group. A recent study in multiple myeloma suggests NKT-cell dysfunction correlates with disease progression [165], suggesting this may be a good disease group to target.

17.5.4
Modulation of Downstream Immune Responses by a-GalCer-activated Cells

A significant feature of NKT cells is their capacity to activate many other effector cell types of the immune system, as was defined in the studies undertaken with α-GalCer activation (Fig. 17.2). The IFN-γ and IL-2 produced by NKT cells during their initial activation, and in conjunction with the IL-12 produced by DC, leads to the enhancement of NK cells and $CD4^+$ and $CD8^+$ T-cell responses [123, 131, 132, 149, 166–171]. In particular, NK cells respond by proliferating, enhancing their cytotoxicity and releasing large quantities of IFN-γ [121, 123, 168, 169]. Indeed, it is the NK-cell-released IFN-γ that ultimately constitutes the bulk of the systemic IFN-γ detected after *in vivo* activation of NKT cells by α-GalCer [121]. One of the best examples of documented cellular responses downstream of NKT cells involves the antitumor activity of α-GalCer [126, 172–178]. The critical involvement of NK cells, IL-12 and IFN-γ in the antitumor activity induced by α-GalCer has now been described in detail [179, 180]. It is believed that the predominant antimetastatic response is not mediated directly by NKT effector cells, but is mediated through the downstream immune responses activated by IFN-γ. Indeed, the control of tumor growth and metastasis by the direct cytotoxic effector function of activated NKT cells remains controversial. *In vitro* studies have demonstrated that NKT cells have the potential to mediate antitumor cytolytic activity [126, 158, 181], and, *in vivo*, it has been demonstrated that IFN-γ released by NKT cells induced the expression of TRAIL on NK cells and correlated this with TRAIL-mediated antimetastatic function [182]. α-GalCer is also protective against primary tumor formation in mice, including those that are spontaneous, carcinogen- or oncogene-induced [183]. NKT cells have been shown to also have a natural role for tumor immune surveillance



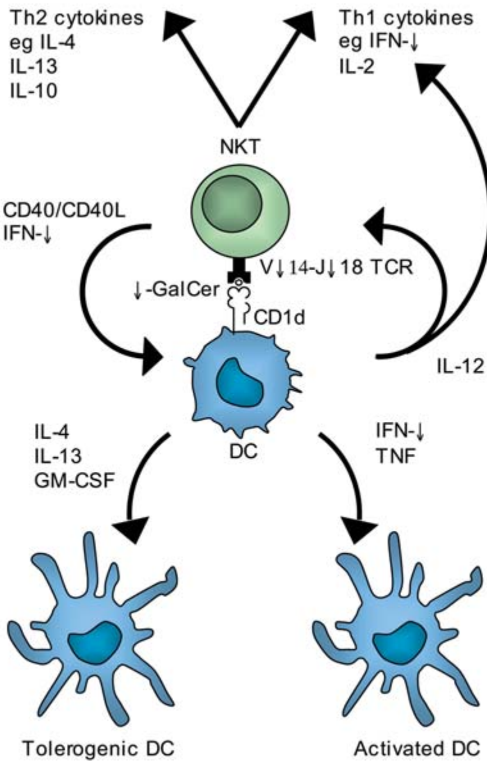


Fig. 17.2 Immunomodulatory functions of activated NKT cells, following recognition of α-GalCer presented by DC. The foreign glycolipid α-GalCer is taken up by DC and presented to the NKT cells. NKT cells receiving an activation signal via TCR ligation will rapidly release predominantly Th1 and Th2 type cytokines. DC receive maturation signals from the activated NKT, including CD40L upregulated on the NKT cell and IFN-γ, resulting in the release of IL-12 by the DC, which principally feeds back to NKT cells to induce a second burst of IFN-γ. Cytokines released by NKT cells (eg. IFN-γ, IL-4, granulocyte colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF), IL-2, IL-10, IL-13) indirectly modulate cellular components of the innate and adaptive immune system, including NK cells, macrophages, $CD8^+$ and $CD4^+$ T cells, and B cells. Immature DC can mature into activated DC or into tolerogenic DC.

as they protect from spontaneous tumors induced by the chemical carcinogen methylcholanthrene [184]. In this instance, protection is critically dependent upon NKT cells, NK cells, $CD8^+$ T cells, CD1d, perforin and IFN-γ [180, 185].

Evidence for a Th2 polarizing response induced by α-GalCer-activated NKT cells comes from studies of B cell activation via IL-4 and possibly CD40/CD40L interactions, resulting in increased B cell CD69 expression, proliferation and production of IgE. Additionally, the presence of α-GalCer activated NKT cells at the time of immunization with an exogenous antigen results in the production of antigen-specific IgE antibodies [150, 168, 186, 187].

Cells belonging to the innate arm of the immune response, such as macrophages, are potentially also activated by NKT-secreted IFN-γ [175, 188]. Importantly, NKT cells activated by α-GalCer have many effects on the DC they interact with, including recruitment [189, 190], initiating the release of myeloid progenitors from bone marrow to the periphery [191] and they have also been shown to kill DC [48, 159] or to mature DC [131, 132].

17.5.5
Adjuvant-like Effect of NKT Cells on DC Mediated Antigen Presentation

The differentiation and activation of DC, mediated by α-GalCer activated NKT cells, [131, 132, 138, 192] potentially influences the subsequent interactions of DC with other cells of the immune system. Indeed, growing evidence supports the adjuvant-like function such DC have on antigen-specific $CD4^+$ and $CD8^+$ T cells undergoing priming for exogenously delivered proteins [131, 132, 167]. The increased T-cell responses result due to the presence of APC such as DC with enhanced function at the time of T-cell priming. The underlying mechanism was recently evaluated, and α-GalCer activated NKT cell-mediated DC maturation could be attributed to TNF-α and IFN-γ release by DC [145]. These cytokines induced enhanced expression of CD80/CD86 co-stimulatory molecules on the surface of the DC [145]. However, CD80/CD86 stimulation signals are not sufficient to induce T-cell immunity to ovalbumin presented on MHC class-I and -II by the DC, and it is the CD40/CD40L signal that is crucial to generate immunity [145]. The signal from the activated NKT cells to the DC appears to be mediated by soluble factors such as IFN-γ and type I IFNs [193]. The adjuvant effect of NKT-cell stimulation is greatly increased if DC are also stimulated through TLR4, suggesting that signals transmitted to DC from the NKT cells can amplify maturation induced by microbial signals [193]. Further experiments have shown that the $CD8^+$ T-cell responses generated by antigen administration in the presence of α-GalCer are cytolytic and can be restimulated using viral vectors [193]. Conversely, the co-administration of a protein antigen with α-GalCer was able to boost a previously primed immune response [193]. These findings suggest that the activation of NKT cells by ligands such as α-GalCer could be exploited to generate potent T-cell vaccines targeted towards the treatment of cancer and infectious agents.

17.6
Role of CD1d-restricted NKT Cells in Disease Models

NKT cells are thought to have an immunoregulatory role *in vivo* (reviewed in Ref. [51]), which is a function controlled by CD1d. Due to their functional diversity, NKT cells have the capacity to either activate or inhibit a wide variety of immune responses, including antitumor and antimicrobial immune responses. Furthermore, in the absence of a microbial or neoplastic trigger, the autoreactive NKT cells can regulate the adaptive immune system to inhibit the development of autoimmunity. The therapeutic potential of the synthetic glycolipid, α-GalCer, due to its immunomodulatory properties as described previously, has been investigated in most disease conditions, including cancer, infection, autoimmune and inflammatory conditions. The natural and activated role of NKT cells in these disease models has been extensively reviewed and will not be discussed further in this chapter [1, 118, 120, 180, 183, 194–200]).

17.7
Conclusions

Great progress has been made in just a few years with respect to understanding the basic molecular events and consequences of NKT cell:APC interactions. α-GalCer has been a wonderfully incisive tool for understanding more about the specific biology of NKT cells. NKT cells may be a promising target for immune-based therapies upon activation via DC because of their potent and progressive cytokine producing ability, and their ability to activate other immune populations in vivo. With further extended studies in humans, we may consider the pharmacological stimulation of Vα24 NKT cells with α-GalCer or other similar glycolipids as a therapeutic modality for a number of immune-based diseases. However, much still remains to be learned concerning the APC that present glycolipid to NKT cells and subsequent activation/regulation of immune responses downstream. The recent discovery of naturally occurring ligands, including the endogenous ligand, iGb3, will surely accelerate our progress.

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18
The Role of Dendritic Cells in T-cell Activation and Differentiation

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18.1
Introduction

Dendritic cells (DCs) are widely accepted as the most potent and versatile antigen-presenting cells (APCs). They have an extraordinary capacity to take up and process antigens for presentation and cross presentation to $CD4^+$ and $CD8^+$ naïve T cells. In addition, DCs express a variety of co-stimulatory molecules and produce several cytokines and chemokines which contribute to shape the quality of the T-cell response generated. In this chapter we will first consider the requirements for T-cell activation and differentiation in terms of signal 1 (peptide-MHC complexes), signal 2 (co-stimulatory molecules) and signal 3 (polarizing cytokines). Then, we will consider the conditions in which DCs fulfill these requirements and how they control the magnitude and class of T-cell responses.

18.2
Requirements for Activation of Naïve T Lymphocytes

Naïve T lymphocytes are resting spore-like cells. To be activated they require a much higher strength of stimulation than effectors and memory T cells. Three factors determine the strength of stimulation that T cells receive: (1) the amount of peptide–MHC complexes, that determines the rate of T-cell receptor (TCR) triggering and initiates signal transduction; (2) the amount of co-stimulatory molecules that engage CD28 and amplify the signaling process; and (3) the duration of the T cell–APC interaction that determines for how long the signaling process is sustained [1]. T cells establish contact with APCs by forming an immunological synapse, where TCRs and CD28 molecules are segregated together in a central area surrounded by a ring of adhesion molecules [2, 3]. In naïve T cells TCRs are inefficiently coupled to downstream signal transduction pathways. Engagement of CD28 by B7 molecules (CD80 and CD86) expressed on APCs recruits membrane rafts containing kinases and adapters to the synapse and amplifies up to 100-fold

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the signaling process initiated by the TCRs [4]. *In vitro* experiments showed that naïve $CD4^+$ T cells require the signal from the TCR to be sustained for several hours in order to be committed to proliferation and to upregulate anti-apoptotic molecules and receptors for homeostatic cytokines (IL-7 and IL-15) [5, 6]. Short TCR signal leads to abortive T-cell proliferation. Co-stimulation via CD28 facilitates T-cell activation by decreasing the time of commitment. In contrast a much shorter time of TCR stimulation even in the absence of CD28-mediated co-stimulation is sufficient for effector T cells to become committed to proliferation and to perform effector function. Based on these findings a quantitative model of T-cell activation has been proposed [7] (Fig. 18.1). According to this model naïve T cells that receive a low strength of stimulation (SoS) (due to low peptide-MHC concentration or avidity, low co-stimulation or transient interaction with APCs) proliferate initially but are "unfit" since they are unable to survive in the absence of antigen. In contrast, naïve T cells that receive higher SoS (due to higher peptide MHC concentration or avidity, co-stimulation and prolonged interaction with APCs) differentiate to effector cells and become "fit" acquiring the capacity to survive in the absence of antigen. Finally, T cells receiving excessive SoS undergo activation-induced cell death (AICD). The SoS model simply accounts for the differential fates of T cells in the immune response, namely tolerance on the one hand and immunity and memory on the other.

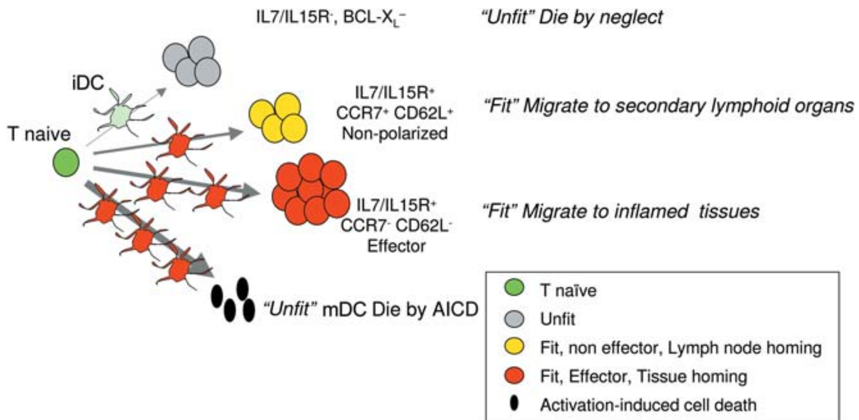

Fig. 18.1 The strength of stimulation (SoS) model of T-cell activation and differentiation. Naïve T cells interacting with antigen-presenting DC receive different SoS (depicted as the length and thickness of the arrow) depending on the DC maturation state the concentration of peptide–MHC and co-stimulatory molecules on DCs and on the duration of T–DC interaction. Short interactions with immature DC (low SoS) are sufficient to induce proliferation, but the proliferating T cells are unfit and do not survive. At higher levels of SoS, proliferating T cells become fit and either remain non polarized of differentiate to effectors under the aegis of polarizing cytokines. Excessive SoS leads to activation induced cell death. iDC, immature DC; mDC, mature DC.

18.2.1 Co-stimulatory and Inhibitory Pathways

The balance between stimulatory and inhibitory signals in T-cell activation is required for effective immune responses to pathogens and for maintaining self-tolerance. Molecules of the CD28 family are key players in these processes since provide second signals that can fine tune T-cell responses (reviewed in [8]). CD28 and ICOS synergize to promote the activation of T-cell responses. However ICOS is not expressed on naïve T cells and an initial TCR stimulation is required to induce its expression indicating that, while CD28 has a predominant role during initial activation of naïve T cells, ICOS may function at later time points in the T-cell differentiation process. Engagement of ICOS on T cells by ICOS ligand on APCs appears to be particularly important for stimulating effector T-cell responses and T-cell-dependent B cell responses [9, 10]. ICOS signaling upregulates Th1 and Th2 cytokines but does not upregulate IL-2 production indicating that ICOS stimulates T-cell effector function but not T-cell expansion. ICOS may have additional functions in the effector phase of the immune response at peripheral sites since ICOS ligand is also expressed on endothelial and epithelial cells [11–13].The tumor necrosis factor family molecule OX40 ligand (OX40L) is a co-stimulatory molecule implicated in many aspect of T-cell response from activation to cytokine production, from migration to B cell help [14–16].

Several receptor–ligand interactions negatively regulate signaling at the immunological synapse. CTLA-4 is the prominent inhibitory receptor expressed on activated T cells and binds to B7.1 and B7.2 with a higher affinity than CD28 [17]. CTLA-4 is present in endosomal structures and it is recruited with a late kinetics to the immunological synapse to extinguish the signaling process [18]. Another inhibitory receptor of the CD28 family is PD-1 [19, 20] that binds two different ligands, PD-L1 which is broadly expressed in lymphoid and non-lymphoid organs and PD-L2 which has a more restricted, but overlapping expression on APCs, The differential tissue distribution of its ligands may explain in part the findings that PD-1 delivers both activatory and inhibitory signals and has distinct biological functions. The new CD28 and B7 family members BTLA, B7-H3, and B7-H4 have more subtle inhibitory functions that may overlap with each another and/or with PD-1 ligands, PD-1, and CTLA-4. It is of note that most of the new B7 family members are present not only on professional APCs but also on various types of tissue cells.

18.2.2 Differentiation to Effector T Cells

Primed T cells that have received sufficient TCR stimulation and co-stimulation, differentiate under the aegis of polarizing cytokines to effector cells that have acquired the capacity to produce effector cytokines and to migrate to inflamed non-lymphoid tissues [21]. In the case of $CD4^+$ T cells IL-12 induces the differentiation towards Th1 effectors that produce IFN-γ and protect from intracellular pathogens,

whereas IL-4 induces differentiation towards Th2 effectors that produce IL-4, IL-5 and IL-13 and protect against extracellular parasites. Th1 and Th2 specific gene expression program is dependent on the expression of the transcription factor T-bet and GATA-3, respectively [22]. These factors function in part through induction of chromatin remodeling at the IFN-γ and IL-4 genes, a process that involves covalent modifications of DNA and histones, such as DNA methylation and histone acetylation at lysine residues [23]. These modifications are epigenetically transmitted throughout cell division and are maintained in memory T cells thus contributing to maintenance of cytokine memory [24]. Besides IL-12 and IL-4, additional cues have been identified that modulate Th1 and Th2 cell differentiation. These include the strength of stimulation and antigen concentration [25, 26], DC–T-cell ratios [27] and co-stimulatory molecules [15, 28, 29]. Recently the Notch ligands Delta-4 and Jagged-1 have been shown to be regulated on DCs by maturation stimuli and to promote Th1 or Th2 differentiation, respectively [30].

Naïve T cells activated in the absence of IL-12 and IL-4 or in the presence of the inhibitory cytokine TGF-β do not differentiate to Th1 or Th2 and remain in a non-polarized state [31–34]. These cells retain the expression of lymph node homing receptors (CD62L and CCR7) and upon adoptive transfer experiments home to secondary lymphoid organs [35].

Differentiation of $CD8^+$ T cells to cytotoxic T cells (CTLs) involves induction of cytokine genes, such as IFN-γ, and cytotoxic molecules such as granzyme B and perforin. Mouse $CD8^+$ T-cell differentiation is promoted by IL-2, while IL-15 expands non-polarized T cells that, upon *in vivo* transfer, home to both lymphoid and non-lymphoid tissues [36, 37].

Besides Th1, Th2 and CTLs there are several other cell fates that can be induced in responding T cells by appropriate external stimuli. These include type 1 regulatory T cells (Tr1) producing IL-10 that are induced *in vitro* by corticosteroids and Vitamin D3 and potently suppress inflammatory responses *in vivo* [38, 39], TGF-β-producing regulatory T cells which are induced by Jagged-1 expressing ACPs [40], and inflammatory T cells which are induced by IL-23, produce high levels of TNF and IL-17 and are implicated in autoimmune inflammatory responses [41].

18.3
Dendritic Cell Maturation

DCs that migrate from tissues to lymph nodes have a life expectancy of a few days and can therefore be viewed as disposable packets, each carrying a given amount of peptide–MHC complexes, co-stimulatory molecules and cytokines. These packets are assembled during the process of DC maturation. DCs can be induced to mature directly by triggering of receptors for microbial products such as Toll-like receptors (TLRs), or indirectly by exposure to inflammatory cytokines or endogenous danger signals released by necrotic cells such as heat shock proteins and urate crystals. DCs maturation can also be induced or reinforced by interaction with cells of the innate and adaptive immune system such as NK cells and NKT on the one

hand and conventional T cells on the other. The maturation program involves the coordinate regulation of approximately 6000 genes that control several DC functions ranging from antigen capture and presentation to co-stimulation, cytokine production and chemokine expression and responsiveness. In maturing DCs the co-stimulatory molecules B7.1 and B7.2 are transported together with MHC class II molecules to the cell surface where they remain associated with membrane microdomains [42], a fact that enhances the effectiveness of TCR and CD28 triggering. While most of the genes appear to be triggered by almost all microbial stimuli some genes are elicited only by specific stimuli while others are induced only in response to combinations of maturation stimuli. The signal-specific maturation programs and the integration of multiple microbial signals by DCs allows a precise discrimination of the context in which antigen is recognized and couples the quality of the adaptive immune response to the nature of the pathogen.

Cytokine and chemokine production by activated DCs is restricted to a temporal window following induction of maturation. For instance, TNF and CCL4 (MIP-1β) are produced early on, between 1 and 3 hours, while IL-12p70 and IL-23 are produced relatively late, between 6 and 16 hours. In general early cytokines can be elicited by a variety of stimuli whereas late cytokines are subjected to a tight regulation since they are effectively produced only in response to combination of maturation stimuli such as multiple TLR agonists and CD40L [43]. Remarkably at late time points DCs lose the capacity to produce IL-12 and become refractory to further stimulation [44]. Consequently the capacity of DCs to prime Th1 and inflammatory T cells is limited to a narrow time window and by a combination of stimuli.

18.4 T-cell Priming by Dendritic Cells

Although the basic principles of DC physiology have been elucidated in considerable details, it is difficult at present to draw a general picture on how antigen presentation is carried out in real life, considering the multiple forms in which the immune system can be challenged by microbial and non-microbial antigens. A first variable to be considered in T-cell priming is the relative contribution to antigen presentation of migrating versus resident DCs. Indeed, some antigens may reach the lymph node directly while others need to be ferried by migrating DCs. This will impact on the frequency and activation state of the antigen-presenting DCs and ultimately on the strength of stimulation that will be delivered to naïve T cells. Antigen-targeting to lymph node resident DCs by coupling it to an antibody to DEC-205 induced tolerance or priming contingent on the simultaneous presence of a DC maturation stimulus [45]. Self-antigens in pancreas constitutively transported by migrating DCs and presented in draining lymph nodes induced abortive T-cell proliferation and establishment of tolerance [46]. In the presence of an infectious agent the same antigen delivered to lymph nodes by high numbers of DCs expressing co-stimulatory molecules and primed for IL-12 production induced productive T-cell activation and differentiation to effector cells. A second variable is the nature

of the activating signals received by DCs. For instance CD40L expressed by specific helper T cells can deliver to DCs a licensing signal for effective CTL priming [47–49]. DCs that have been directly activated by microbial products possess superior T-cell stimulatory capacity as compared to those that have been activated in a bystander fashion by inflammatory cytokines [50]. The third variable is the kinetics of DC activation. Recently migrating DCs that secrete Th1 polarizing cytokines will be capable of driving Th1 while the same DCs at later time points, having exhausted IL-12 producing capacity, will prime T cells that either develops towards Th2 or remain non-polarized [44].

18.4.1
Priming of Th1 and Inflammatory T-cell Responses

The differentiation of naïve $CD4^+$ T cells towards IFN-γ-producing Th1 is promoted by IL-12 whereas differentiation towards IL-17-producing inflammatory T cells is promoted by IL-23. The heterodimeric cytokines IL-12 and IL-23 are produced by DCs primarily in response to specific combinations of stimuli. The highest production is elicited by triggering DCs first with microbial stimuli and then with CD40L, while each trigger alone provides an insufficient stimulus [43, 51]. Synergistic stimulation of TLR3 or TLR4 together with TLR8 results in over induction of IL-12, IL-23 and Delta-4 leading to DCs with enhanced and sustained Th1-polarizing capacity [52]. The simultaneous triggering of different TLRs on DCs may provide a "combinatorial security code" for the induction of inflammatory T-cell response. In vaccine settings appropriate synergic combinations of TLR agonists may turn out to be superior adjuvants for the induction of IL-12 dependent immune responses.

IFN-γ has also Th1 polarizing capacity and is produced not only by effector T cells but also by NK cells. Mouse experiments indicate that mature DCs that migrate to lymph nodes induce a rapid recruitment of NK cells and that the IFN-γ produced by NK cells is necessary for efficient Th1 polarization *in vivo* [53]. The role of NK cells in human Th1 responses remains to be determined but IFN-γ-producing NK cells capable of directly stimulating DCs have been found in human secondary lymphoid organs [54, 55].

18.4.2
Priming of Th2 Cells

Th2 polarization is primarily driven by IL-4, but the source of this cytokine during T-cell priming remains to be determined. While one study suggested that DCs produce IL-4 when stimulated by *Candida hyphae* [56], other studies indicate that DCs under appropriate stimulatory conditions can effectively prime Th2 responses in spite of not being able to make IL-4. IL-6 produced by DCs has been reported to be a Th2 polarizing signal [59]. Human DCs primed with thymic stromal lymphopoietin (TSLP), a cytokine produced by keratinocytes and other epithelial cells of mucosal lymphoid tissues and thymus, induce the differentiation of $CD4^+$ and $CD8^+$ naïve T cells into proallergic effectors [57, 58]. DCs with Th2 polarizing capacity

can be generated either by maturation stimuli that do not induce IL-12 or by DCs that have exhausted IL-12 producing capacity (i.e. more than 24 hours after induction of maturation). One possibility is that the IL-4 required for Th2 polarization is produced by T cells themselves. Indeed, naïve T cells upon prolonged stimulation can produce low amounts of IL-4 that is sufficient to promote their own differentiation towards Th2. In conclusion it is possible that Th2 differentiation would simply result from a lack of Th1 polarizing cytokine.

18.4.3
Imprinting Tissue Homing Receptors

Besides acquiring cytokine producing capacity, Th1 and Th2 cells also acquire new patterns of chemokine receptors and integrins that are instrumental for their subsequent migration to effector sites [60]. Upon interaction with mature DCs, proliferating T cells rapidly upregulate CXCR3 and CCR4 that are maintained at high levels on Th1 or Th2 cells, respectively. The Th1-associated receptors CCR5 and CXCR6 and the Th2-associated receptors CCR3 and CRTh2 are selectively up-regulated in Th1 or Th2 cells only at late cell divisions or after repeated restimulation [61, 62]. The polarizing cytokines IL-12 or IL-4 are sufficient to instruct chemokine receptor expression in activated T cells. In addition, the acquisition of some tissue specific homing receptors is regulated by additional signals delivered by DCs. Thus, ligation of OX40 on $CD4^+$ T cells by OX40L expressed on DCs leads to upregulation of CXCR5, the receptor for homing to B cell follicles [15]. DCs from Peyer's patches produce retinoic acid and induce expression on activated $CD8^+$ T cells of $\alpha4\beta7$ and CCR9, the receptor for the gut-associated chemokine CCL25 (TECK), whereas reciprocally skin Langerhans cells induce CLA and CCR4, the receptor for CCL17 (TARC) and CCL22 (MDC) [63–65].

18.4.4
The Role of Plasmacytoid Dendritic Cells in T-cell Responses

A distinct subset of DCs called plasmacytoid DCs (pDCs) or interferon-producing cells (IPCs) has been described in humans and, more recently, in mice [66]. Although pDCs are capable of presenting endogenous antigens to $CD8^+$ T cells, their hallmark function is the production of high amounts of type I interferons (IFNs) following viral infection or TLR7 or TLR9 triggering by specific agonists. Besides their direct antiviral activity IFNs are endowed with potent immunomodulatory functions and act as powerful DC maturation stimuli and may therefore contribute to a local milieu that is conducive of T-cell priming [67]. Recently in a model of cutaneous *Herpes simplex* virus (HSV) infection it has been shown that pDCs fail to induce CTLs but create a local immune field that helps lymph node DCs to induce protective antiviral CTL responses [68].

Initial studies suggested that human pDCs upon prolonged *in vitro* stimulation with CD40L acquire Th2 polarizing capacity [69] while subsequent studies indicated that upon viral stimulation they are capable of priming Th1 responses [70], al-

though they do not appear to be an abundant source of IL-12. Mouse pDCs, cultured from bone marrow precursors or isolated from spleen, can induce the development of both Th1 and Th2 effector cells depending on the dose of antigen [71]. Thus, as observed in the case of conventional DCs, antigen dose, nature of maturation stimuli and state of pDC maturation will determine whether a Th1 or Th2 response develops.

18.5 Concluding Remarks

In this chapter we have attempted to dissect the variables that influence the outcome of DC–T-cell interaction. It is worth considering that the immune response results from cellular interactions and it is a process extremely dynamic and stochastic and therefore difficult to model. Besides the variables considered above, it is important to consider that T cells compete for access to DCs. This may be particularly true in the late phases of an immune response when large numbers of proliferating cells may limit the stimulation of newly incoming cells. Because of the stochastic nature of DC–T-cell interaction, proliferating T cells even within the same clone may receive different strength of TCR and cytokine stimulation, a fact which may lead to intraclonal diversification. This has been suggested to be a mechanism responsible for the generation of functionally heterogeneous populations of memory T cells within the same clone [72].

The scenarios depicted above are now being tested using novel real-time imaging technologies that have begun to measure rate directionality of T-cell movement relative to antigen-presenting DCs in lymph nodes and the duration of dynamics of cell to cell contacts, shedding light on the earliest events in T-cell activation in a physiological setting [73–77]. These studies are expected to provide invaluable information on how DCs control T-cell immunity and may hold the key to improved strategies for vaccination and immunotherapy.

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19
Cytokines Produced by Dendritic Cells

David F. Tough

19.1
Introduction

Dendritic cells (DC) are key regulators of the immune system, serving as a link between the innate response to infection and the generation of adaptive immune responses. One mechanism that appears to play an important role in the immunoregulatory activity of DC is the expression of cytokines. Production of cytokines by resting DC is thought to contribute to normal homeostasis, whereas cytokines expressed by activated DC may contribute to many aspects of the response to infection, including the stimulation of innate effector mechanisms and the initiation and polarization of the immune response. Different DC subpopulations exhibit distinct capacities for cytokine production, which may be linked with specialized roles in the immune system. This chapter will review a subset of the cytokines reported to be expressed by DC, and discuss the way in which DC-expressed cytokines fit with the function of these cells.

19.2
DC Cytokine Expression: A Few Caveats

Although DC have been reported to express many different cytokines, these findings need to be interpreted with some caution for several reasons. Firstly, most investigations of DC cytokine expression have been conducted *in vitro*, and typically require that DC are placed in tissue culture for a period of time. How well the results from such studies reflect the *in vivo* situation is unclear, since the environment of a DC is likely to be a critical factor in determining its overall program of gene expression. For instance, it is well recognized that DC isolated from mouse lymphoid tissues undergo "spontaneous" maturation when cultured *in vitro*. Hence, measurement of DC cytokine production *in vitro* may lead to a misleading view of basal cytokine expression patterns. The problem may be lessened, to a certain extent, by examining cytokine expression at the mRNA level immediately after

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DC purification. This latter approach, however, has the disadvantage of failing to demonstrate directly that the cytokines are expressed at the protein level and secreted. Moreover, the process of extracting the cells from their natural environment and subjecting them to purification procedures may, in itself, alter the expression of cytokine genes.

Secondly, provision of definitive evidence that a cytokine has been produced by a DC can be difficult. Often, cytokine production by "purified" DC is measured at the population level, for example, by measuring cytokines present in the supernatant of cultured cells. In such assays, it is difficult to rule out the possible presence of minor contaminating cell types capable of high cytokine expression. More convincing evidence can be provided by examining cytokine expression at the single cell level (for example, by combining cell surface staining for DC markers with intracellular staining for cytokines). Even this approach, however, can be complicated by the expression of DC markers (for example CD11c) by other cells types [1].

Finally, data on DC cytokine production frequently derives from experiments on DC that were generated from precursor cells *in vitro*: either human monocyte-derived DC or mouse bone-marrow-derived DC (BMDC). These *in vitro*-generated DC represent useful models because of the ease with which large populations of DC can be produced and because of the relative resistance of these cells to the "spontaneous" maturation that affects *in vivo*-generated DC. However, it is evident that the cytokine environment in which these cells are derived can affect their cytokine production. In addition, it is unclear whether the *in vitro*-derived DC have any exact *in vivo* equivalents.

Notwithstanding these caveats, there is persuasive evidence that DC are able to produce a number of key immunoregulatory cytokines. In the examples described below, cytokine expression has typically been shown for DC from diverse sources and under numerous different conditions, lending strong weight to the conclusion that these cytokines are actually produced by DC. Furthermore, an emphasis is placed on cytokines for which expression is elicited following exposure of DC to maturation- or activation-inducing stimuli, leaving aside the less well resolved issue of basal cytokine expression.

19.3 Type I Interferon

Type I interferon (IFN-α/β), which comprises a large family of closely-related genes, plays a crucial role in the innate defense against virus infection [2–7]. Triggering of the IFN-α/β receptor (IFN-α/βR) initiates the expression of a variety of molecules that can inhibit virus replication at multiple stages of the viral life cycle [8, 9]. In addition, IFN-α/β has also been shown to exert immunomodulatory activity, possessing the ability to stimulate DC and promote immune responses [10]. Expression of IFN-α/β is induced rapidly by viral infection, and also by various components of infectious agents, including LPS, bacterial DNA, double- and single-stranded RNA, viral glycoproteins and bacterial proteins [9, 11–25].

While most cell types seem to be capable of expressing IFN-α/β in response to virus infection, a subpopulation of DC precursors, the plasmacytoid DC (PDC), has been shown to secrete particularly large quantities of IFN-α/β [26–29]. PDC are equivalent to the "natural interferon-producing cells" present in human blood, and have now been identified in many different species [30–34]. PDC secrete IFN-α/β in response to various stimuli, including viruses, oligodeoxynucleotides containing immunostimulatory motifs (CpG ODN), Toll-like receptor 7 (TLR7) agonists and CD40L + IL-3 [24–29, 35–39]. Each of these stimuli also trigger the maturation of PDC into cells with many properties of DC. These "mature PDC" possess the ability to stimulate and polarize T-cell responses, although their precise role as antigen-presenting cells remains to be defined.

In addition to PDC, other DC have also been shown to produce IFN-α/β, although this is typically at lower levels than for PDC. DC reported to express IFN-α/β include: human peripheral blood $CD11c^+$ DC after exposure to the synthetic dsRNA poly IC; human monocyte-derived DC treated with poly IC, lipopolysaccharide (LPS), influenza virus, activated T cells or *Mycobacterium tuberculosis*; mouse Langerhans cells (LC) treated with poly IC; mouse BMDC treated with poly IC or virus-like particles; and mouse splenic DC exposed to viruses, LPS or poly IC [1, 40–50]. Of note, mouse BMDC and splenic DC have been shown to produce very high amounts of IFN-α/β (equivalent to PDC, on a per-cell basis) when poly IC is transfected directly into the cytoplasm [47]. This may be indicative of a response of DC to direct viral infection.

DC-secreted IFN-α/β is likely to play an important role in the innate antiviral response, although the specific contribution of PDC and conventional DC to overall IFN-α/β production will vary for different viruses [45]. Since both PDC and DC localize in lymphoid tissues after contact with maturation-inducing stimuli, IFN-α/β produced by these cells may contribute in particular to the protection of immune cells from viral infection and destruction. In addition to its antiviral effects, IFN-α/β can act in several ways to stimulate and modulate the generation of immune responses, including stimulating DC maturation, inducing expression of chemokines and cytokines, promoting B-cell differentiation into plasma cells and regulating T-cell differentiation and survival [50–77]. *In vivo*, IFN-α/β has been shown to promote antibody, $CD4^+$ and $CD8^+$ T-cell responses [55, 78–83]. Thus, production of IFN-α/β by DC will contribute to both innate and adaptive immunity.

19.4
IL-12, IL-23, IL-27

IL-12, IL-23 and IL-27 are three structurally-related members of the IL-12 family of cytokines [84]. Each is a heterodimer, consisting of an alpha helical subunit (p35, p19 or p28) combined with a soluble receptor-related subunit (p40 or Epstein Barr virus-induced protein 3 (EBI3)); functional IL-12 is formed by p35 and p40, IL-23 by p19 and p40, and IL-27 by p28 and EBI3. The three cytokines bind to distinct receptors, with the receptors for IL-12 and IL-23 sharing a common subunit. All

three family members are able to promote IFN-γ production by T cells, suggesting that they may play a role in driving Th1-type responses, although the specific contribution of each cytokine is still unclear [84]. In this respect, it is notable that IL-12, IL-23 and IL-27 are active on T cells at different stages of differentiation. IL-12 stimulates proliferation and IFN-γ production by both naïve and memory $CD4^+$ T cells, while IL-27 and IL-23 selectively activate naïve and memory T cells respectively.

It has been demonstrated that IL-12, IL-23 and IL-27 can all be expressed by DC in response to maturation-inducing stimuli. IL-12 expression has been the most extensively studied, and is often used as a correlate of DC maturation. Expression of IL-12 has been described for human monocyte-derived DC, human peripheral blood DC, mouse BMDC, human and mouse PDC, human and mouse LC and DC isolated from human and mouse lymphoid tissues (for examples, see [32, 85–100]). Stimuli shown to induce IL-12 expression include intact viruses and bacteria, LPS, bacterial toxins, CpG ODN, poly IC, TLR7 agonists, kinin peptides, TNF-α plus IL-1, bacterial lipoproteins and lipopeptides, protozoal components, heat shock proteins, high mobility group box protein 1 (HMGB1), TRANCE and ligation of CD40 [44, 61, 87, 90–92, 95, 97, 99, 101–114]. Here, it should be noted that specific stimuli vary in the potency with which they can induce IL-12 expression by particular DC types, and that the ability of DC to produce IL-12 can be up- or downregulated by exposure to other factors. For instance, expression of IL-12 in response to CD40 triggering appears to be dependent on priming of DC by a microbial signal [105, 115], while the amount of IL-12 expressed by stimulated DC can be modulated by several cytokines, including IL-4, IL-10, IL-13, GM-CSF, IFN-γ, CCL2, TGF-β and IFN-α/β [64, 89, 102, 108, 116–125]. The potential for IL-12 expression can also be influenced by the methods used for cell purification [126].

DC expression of IL-12 is thought to make an important contribution to the production of Th1-type responses [85, 87, 88, 91, 104, 109, 127–130]. *In vitro*, neutralization of IL-12 can abrogate the ability of DC to promote the generation of IFN-γ expressing T cells [85, 87, 101], although IL-12-independent mechanisms have also been observed [109]. Likewise, adoptive transfer experiments have shown that the ability of injected DC to induce Th1 responses *in vivo* correlates with their production of IL-12, and that IL-12-deficient DC are defective in their ability to stimulate Th1 responses [129–131].

Polarization of immune response by DC may be partially linked to interactions between DC and NK cells. In response to various maturation-inducing stimuli, DC can stimulate NK cells to express IFN-γ and cytolytic activity [1, 45, 132–152]; under some conditions, NK-cell activation is dependent on DC production of IL-12 [134, 135, 145, 146, 149, 151]. Notably, the interaction between NK cells and DC is reciprocal, with NK cells enhancing functions associated with DC maturation, including the production of IL-12 [137, 143, 144]. Thus, IL-12 and IFN-γ elicited through DC–NK cell interaction may contribute to the generation of Th1 and $CD8^+$ T-cell responses [143]. In addition to subsequent effects on the adaptive immune response, DC activation of NK cells represents an important innate pathway for host defense against pathogens [135, 153].

Much more limited information is available with regard to IL-23 and IL-27 expression by DC, although activated DC were demonstrated to be significant producers of both during the initial characterization of these cytokines [154, 155]. Expression of IL-23 has been reported for: mouse and human peripheral blood-derived DC stimulated to mature with a combination of TNF-α, LPS and anti-CD40; human monocyte-derived DC treated with CD40L, cytokine cocktails (TNF-α, IL-1β, IL-6, prostaglandin E2) or gram-negative or gram-positive bacteria; and mouse BMDC-treated with prostaglandin E2 [154, 156–161]. In addition, *in situ* expression of IL-23 by DC has been detected in dermal lesions of patients with psoriasis vulgaris and in the lamina propria of the mouse small intestine [157, 162]. Human monoctye-derived DC have been shown to express IL-27 in response to a number of stimuli, including a combination of CD40L, IFN-γ and LPS or *Staphylococcus* enterotoxin A (SEA), gram-negative and gram-positive bacteria, LPS and poly IC [70, 155, 158, 159, 161].

It should be noted that the expression patterns of IL-12, IL-23 and IL-27 by DC are not identical. For instance, the expression of IL-27 appears to precede that of IL-12, at least at the mRNA level [155], while some stimuli that are able to induce IL-12 expression fail to trigger expression of IL-23 or IL-27 [158]. Moreover, opposing effects of particular stimuli on DC expression of IL-23 vs IL-12 and IL-27 expression have been reported [70, 158]. These data imply that the three cytokines are likely to have unique roles in regulating the immune response, in keeping with their different target cells and functional effects [84].

19.5 IL-18

IL-18 is a member of the IL-1 superfamily that plays a role in the induction of innate immunity and in the regulation of Th1 and Th2 responses [163–165]. Like IL-1β, IL-18 is synthesized as an inactive precursor lacking a typical signal peptide and requires caspase-1 for cleavage and release of the mature, active form of the protein [166]. IL-18 shares functional properties with IL-12, including the ability to activate NK cells and to stimulate proliferation and IFN-γ production by activated T cells [163, 167–172]; many of the immunoregulatory activities of IL-18 are mediated in synergy with IFN-α/β or IL-12 [1, 54, 169, 170, 173–178].

Human monocyte-derived DC have been shown to produce IL-18 in response to various stimuli, including lipoteichoic acid, CD40L and IFN-γ (± SEA), bacterial infection and inflammatory cytokines [43, 70, 152, 156, 179–184]. *In vitro* expression of IL-18 has also been reported for enriched populations of mouse LC, human *in vitro*-derived LC and mouse LN DC [152, 185–189]. *In situ*, IL-18 protein has been detected in a population of DC expressing ICAM-3 grabbing nonintegrin (DC-SIGN) in the colonic mucosa of patients with Crohn's disease [190].

Because of its synthesis as an inactive, leaderless precursor, functional IL-18 expression may be regulated at several steps. Interestingly, human monocyte-derived DC matured with LPS have been shown to accumulate intracellular pro-IL-18, which is secreted in active form upon DC presentation of antigen to T cells [179, 187, 191].

IL-18 secreted by DC has been shown to act synergistically with IL-12 in stimulating the production of IFN-γ by T cells and the development of Th1 cells *in vitro* [185, 186]. Likewise, DC-produced IL-18 can contribute to NK-cell activation in combination with IL-12 and/or IFN-α/β [1, 134]. Notably, DC also express the IL-18R, and IL-18 has been reported to act as a chemoattractant and a maturation factor for human DC [192–194].

19.6 IL-6

IL-6 is a highly pleiotropic cytokine with the ability to affect the function of many cells of the immune system [195–206]. Expression of IL-6 is one of the most common and sensitive responses of DC to maturation/activation stimuli. IL-6 has been reported for: DC isolated from mouse spleen, LN, lungs or Peyer's patches; mouse BMDC; human DC generated from monocytes, adherent PBMC or cord blood $CD34^+$ progenitors; DC in human peripheral blood; mouse and human LC; human, mouse and rat PDC; porcine monocyte-derived DC; and bovine afferent lymph DC [34, 37, 93, 101, 189, 207–219]. Depending on the DC type, IL-6 expression can be upregulated by LPS, peptidoglycan, CpG ODN, poly IC, TLR7 agonists, viral or bacterial infection, virus-like particles, heparin sulfate, chemical irritants, HMGB1, or triggering through CD40, OX40L, TRANCE-R or CD80/86 [95, 101, 107, 113, 184, 209, 210, 212, 213, 219–230].

IL-6 has the potential to impact on many different aspects of the immune response, given the diverse activities of this cytokine. IL-6 is well recognized as a stimulator of both T and B cells, and can also affect DC function, contributing to DC maturation and altering antigen processing [195–206]. Although the specific role of DC-produced IL-6 remains to be fully elucidated, *in vitro* studies imply an important function. Of particular interest, secretion of IL-6 by DC was shown to control the activity of $CD25^+$ $CD4^+$ regulatory T cells (Tregs) *in vitro*; these effects appear to include both inhibiting the suppressor function of the Tregs and reducing the ability of activated T cells to respond to Tregs, with the net effect of strongly potentiating the T-cell response [231–233]. Secretion of IL-6 by DC has also been shown to promote the generation of Ig-secreting B cells. This has been observed for PDC and Peyer's patch DC when mixed with B cells *in vitro* in the presence of appropriate stimuli [217, 234]. In addition, it has been proposed that the expression of IL-6 by pulmonary DC plays a role in inhibiting the generation of Th1 responses in the lung [216].

19.7 IL-1

IL-1 is a major pro-inflammatory cytokine that has multiple activities within the immune, endocrine and neuronal systems [235]. IL-1 consists of two molecular

species, IL-1α and IL-1β, which exert similar biological activities through the type 1 IL-1R [236]. Expression of IL-1 is controlled at several levels and is independently regulated for IL-1α and IL-1β; for IL-1β, this includes both transcriptional and translational regulation [237]. Both IL-1α and IL-1β are synthesized as precursors that lack leader peptides. Notably, pro-IL-1α is fully active while pro-IL-1β is largely inactive. Active IL-1β is released from the cell after cleavage of the precursor protein by caspase-1, although extracellular proteases can also cleave and activate IL-1β [238, 239]. IL-1α but not IL-1β exists in a myristoylated form that can be presented on the cell surface [238].

Amongst *in vivo* generated DC, LC represent the cell population that has been most extensively studied with respect to IL-1 expression. IL-1β mRNA has been detected in LC isolated directly from the skin of normal mice, and expression of both IL-1α and IL-1β mRNA has been observed in $CD11b^+$ DC isolated from skin draining LN (note that $CD11b^+$ DC include immigrant LC as well as dermal DC [240]) [207, 241, 242]. Higher levels of IL-β mRNA expression have been observed in experiments where LC were placed in culture or when LC were obtained from mice infected with herpes simplex virus-1 (HSV-1) or *Leishmania major* [207, 242, 243]. Similarly, human LC isolated from epidermal cell preparations have been shown to express IL-1β and IL-1α mRNA after stimulation with phorbol myristate acetate (PMA), although secretion of protein was not detected [244]. Intracellular IL-1α and IL-1β protein have been detected in human LC after stimulation with LPS *in vitro*, and in the dermis of individuals undergoing a delayed-type hypersensitivity reaction after injection of tuberculin-purified protein derivative (PPD) [214, 245]. Other *in vivo* derived DC that have been reported to express IL-1 include: human peripheral blood DC, which have been demonstrated to upregulate IL-1α mRNA following activation with PMA and to secrete IL-1α and IL-1β in response to stimulation with viruses *in vitro*; a subpopulation of DC found in bovine afferent lymph; and human thymic DC, which were shown to secrete a small amount of IL-β in response to stimulation with LPS [211, 218, 220, 246].

A number of studies have investigated IL-1 expression by DC generated precursor cells in tissue culture. These include *in vitro*-derived human and mouse LC, which were shown, respectively, to upregulate IL-1β mRNA in response to chemical irritants and to secrete IL-1α after LPS treatment [247, 248]. For mouse BMDC, increased expression of IL-1α and/or IL-1β mRNA has been observed after phagocytosis of particulate matter, ligation of TRANCE or CD40, or treatment with LPS [86, 95, 107]. In addition, BMDC have been shown to secrete IL-1 in response to Fas ligation or exposure to LPS, heparin sulfate, or a synthetic derivative of the macrophage-activating lipopeptide (MALP-2) from *Mycoplasma fermentans* [223, 248–251]. Human DC generated from peripheral blood precursors have also been shown to express and secrete IL-1α and IL-1β in response to various stimuli [184, 189, 214, 252–254]. Notably, these studies indicated that regulation of IL-1 expression by DC is complex. In one example, secretion of bioactive IL-1β was shown to require two signals [252]. The first could be triggered by inflammation or CD40L-mediated contact with $CD4^+$ T cells and induced intracellular accumulation of pro-IL-1β, while the second, which can be provided by activated $CD8^+$ T cells, stimulat-

ed secretion of bioactive IL-1β. In another study, DC expressed pro-IL-1β intracellularly in response to LPS or viable or inactivated *Chlamydia trachomitis* bacteria, but active IL-1β was only secreted in response to infection with viable bacteria (associated with activation of caspase-1) [184].

Although the conditions under which IL-1 is actually secreted by DC *in vivo* remain to be established, production of this cytokine could potentially contribute to DC function. This may include effects on T-cell activation. For instance, IL-1β produced by LPS-treated BMDC has been shown to contribute to a reversal of the "anergic" phenotype exhibited by Treg cells, while the ability of the MyD-1$^+$ but not MyD-1$^-$ subpopulation of DC in bovine afferent lymph to stimulate CD8$^+$ T-cell proliferation *in vitro* is linked to the ability of the former but not the latter DC to produce IL-1α [233, 255]. Moreover, greater production of IL-1 by DC from C57BL/6 mice than BALB/c mice appears to play a role in the selective generation of a protective, Th1 response after infection with *Leishmania major* [242, 248].

IL-1 may also have autocrine/paracrine effects on DC themselves, since IL-1 can act together with other inflammatory stimuli to induce maturation of DC, including LC [250, 256–265]. In addition to promoting DC maturation, IL-1 has been shown to stimulate the migration of LC from the epidermis to the draining LN [266–269].

19.8 TNF-α

TNF-α is a powerful pro-inflammatory cytokine that has multiple effects in regulating innate and adaptive immunity [270]. Expression of TNF-α is regulated at both the transcriptional and post-transcriptional levels [271]. The TNF-α protein is synthesized as a precursor that is initially displayed on the plasma membrane before being released as a soluble monomer through the action of matrix metalloproteases [272].

Similar to IL-6, expression of TNF-α by DC appears to be a relatively common response to maturation/activation stimuli. TNF-α production has been reported for: *in vivo* or *in vitro* generated LC; human peripheral blood DC; human DC derived *in vitro* from monocytes, adherent PBMC or CD34$^+$ progenitors; mouse bone marrow-derived DC; rhesus macaque monoctye-derived DC; human and porcine PDC; and mouse and rat splenic DC [24, 27, 93, 101, 189, 211, 213, 221, 245, 273–278]. Stimuli shown to induce TNF-α expression by DC include: various viruses or virus like particles; crosslinking of CD40, OX40L, Fas or lymphocyte activation gene-3 (LAG-3); chemical irritants; bacterial proteins; LPS; oxidative stress; CpG ODN; TLR7 ligands; prostaglandins; heparin sulfate; heat shock proteins; peptidoglycan; bacteria; HMGB1; and interaction with activated NK cells [97, 101, 113, 114, 151, 184, 212, 213, 222, 223, 226, 227, 229, 243, 249, 253, 274–276, 279–285] In addition, TNF-α has been detected *in situ* in human LC at the site of PPD-induced DTH reactions, and in DC infiltrating pancreatic islets in the non-obese diabetic mouse [245, 286].

Interestingly, there is some evidence that, analogous to the unique capacity of PDC to secrete large amounts of IFN-α/β, particular DC subpopulation(s) may be specialized for high production of TNF-α. Thus, the monoclonal antibody M-DC8 was shown to identify a DC subpopulation present in human peripheral blood which comprises <1% of PBMC and produces much higher quantities of TNF-α than either monocytes or other DC in the blood when stimulated with LPS [287]. These M-DC8$^+$ DC seem to be associated with inflammatory responses, as they have been identified in the T-cell area of inflamed tonsils and in the inflamed ileal mucosa in Crohn's disease [288]. In mice, a subpopulation of CD11b^{int} DC expressing high amounts of TNF-α was shown to be recruited to the spleen following infection with *Listeria monocytogenes* [284]. TNF-α production by these DC appears to be important for innate immune defense against this bacterium, since CCR2$^{-/-}$ mice failed to recruit these cells to the spleen and were unable to clear the primary infection.

TNF-α secreted by DC would be expected to contribute to inflammation and activation of innate immunity. In addition, TNF-α is known to affect DC in various ways, implying that production of this cytokine may serve an autocrine or paracrine function. *In vitro*, TNF-α has been shown to enhance the viability of LC, and in combination with other stimuli to both promote the development of DC from precursors and to stimulate DC maturation/activation [27, 32, 201, 260, 262, 274, 275, 289–300]. TNF-α has also been shown to play a role in DC activation *in vivo* [301]. Moreover, TNF-α induces the migration of DC from peripheral tissues to the draining LN [259, 266, 302–307].

19.9 Concluding Remarks

There is abundant evidence that DC can produce various cytokines. In addition to those discussed here, DC have also been reported to express several other factors, including IL-2, IL-10, IL-13, IL-15, IL-16, IFN-γ, a proliferation-inducing ligand (APRIL), B-cell activating factor belonging to the TNF family (BAFF) as well as numerous chemokines. The capacity to produce these cytokines is in keeping with the key role that DC play in regulating the immune response, since the range of cytokines expressed can impact on many aspects of innate and adaptive immunity.

As briefly touched upon in this review, there is specialization in the production of cytokines by DC, which is evident in two ways. Firstly, particular DC subpopulations appear to possess unique capacities for the expression of certain cytokines, with the ability of PDC to manufacture large quantities of IFN-α/β being the most prominent example. Secondly, different DC subpopulations produce cytokines in response to distinct stimuli. This selective responsiveness is linked to the expression of pathogen receptors, which allow DC to recognize various components of microorganisms. Unequal distribution of pathogen receptors is best characterized for the Toll-like receptor family, members of which are differentially expressed among DC subpopulations [42, 99, 226, 308–310]. As DC heterogeneity continues

to be uncovered, it seems likely that further specialization in cytokine production will be revealed. It will be important to understand how differential cytokine expression contributes to the function of individual DC subsets.

Given that all of the cytokines described in this review can also be produced by other cell types, a question remains as to the specific role of cytokine production by DC. The contribution of DC to the induction of inflammation and stimulation of innate immunity probably overlaps considerably with that of other cytokine-secreting cells, such as monocytes and macrophages, although differences in anatomical distribution might confer dedicated functions to particular cells in specific niches. Conversely, DC production of cytokines is more likely to play a unique role during the process of antigen presentation and T-cell activation. In this respect, directed secretion of cytokines at the site of interaction between DC and T cells (the immunological synapse) may be relevant [311]. Future studies aimed at studying such localized delivery of cytokines as well as cytokine expression *in vivo* will help to elucidate the importance of DC as cytokine producers.

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VII
Th1 and Th2 Decision

20
The Plasticity of Dendritic Cells Populations in Promoting Th-cell Responses

André Boonstra, Giorgio Trinchieri and Anne O'Garra

An effective immune system requires a high degree of flexibility in order to combat different types of pathogens. In peripheral tissues, dendritic cells (DC) sample their environment for antigens and transport them to the lymph nodes. The close interaction between DC and naïve helper T (Th) cells in the lymph nodes results in the activation of Th cells via the interaction of antigen complexed with MHC II on the antigen-presenting cell and the specific T-cell receptor on Th cells, leading to the development of appropriate effector Th-cell responses [1–3]. As will be discussed in more detail below, DC do not merely provide an "on/off"signal, but exhibit a remarkable plasticity in directing the development of Th1 or Th2 responses. This flexibility is the net result of the existence of different DC populations, their modulation by environmental constraints and conditions, their response to pathogens, their past experiences, and the differential state of maturation. Distinct DC populations may be poised to respond to different pathogens due to differential Toll-like receptor (TLR) expression. Thus, DC, because of their heterogeneity and plasticity, are able to instruct the immune system to tailor its responses to deal with a wide range of pathogens efficiently.

20.1
Effector Th-cell Populations

Activation of naïve T cells by antigen presenting cells can result in the development of Th1 or Th2 cells, which can be distinguished on the basis of the cytokines that they produce. Th1 cells produce interferon (IFN)-γ and lymphotoxin and play a central role in cell-mediated immunity important for the eradication of intracellu-

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lar pathogens, such as bacteria and viruses [4, 5]. Th2 cells, producing interleukin (IL)-4, IL-5, and IL-13, are important in immunity to helminths, and contribute to eosinophilic inflammation and allergic reactions [6, 7].

The cytokines produced in the micro-environment are key factors in determining the type of Th response. The dominant cytokines are IL-12 and IL-4, which induce Th1 and Th2 responses, respectively [8, 9]. Mice deficient in IL-12, or in the IL-12-induced transcription factor STAT-4 had markedly reduced Th1 responses [10, 11], whereas mice deficient in IL-4 or in IL-4-induced STAT-6 had reduced Th2 responses [12–16]. Th1-produced IFN-γ and Th2-produced IL-4 can promote the growth or differentiation of their own respective T-cell subsets by upregulating the transcription factors T-bet and GATA-3, respectively [17–22], but they can also antagonize the development of the opposing subset [23]. For example, IL-4 directly triggers the differentiation into Th2 cells and downregulates their expression of the IL-12-receptor β2 chain [24], whereas IFN-γ upregulates the expression of the IL-12-receptor β2 chain on Th1 cells in mice [24]. Species-specific differences are observed with respect to the effects of type I IFN. IFN-α and IFN-β upregulate the expression of the IL-12-receptor β2 chain, thereby enhancing IL-12-mediated Th1-cell development in human T cells [25]. Moreover, type I IFN has been reported to induce human T cells – but not mouse T cells – to differentiate into Th1 cells even in the absence of IL-12 [26]. The basis of the species-specific activity of type I IFN is unclear, because type I IFN transiently induces STAT-4 phosphorylation both in human and mouse T cells, although the effect may only be observed at higher type I IFN concentrations in the latter [27]. Indeed, LCMV infection *in vivo* results in STAT-4 activation by IFN-α/β in murine T cells and consequently IFN-γ production. Interestingly, IFN-α/β-induced activation of STAT-1 was shown to inhibit STAT-4 phosphorylation and IFN-γ production [27]. Therefore, the relative levels of activated STAT-1 versus STAT-4 by IFN-α/β may determine the IFN-γ production during viral infection.

20.2 Factors Inducing the Development of Th1 or Th2 Cells

DC are known to induce the activation and proliferation of T cells. Moreover, the finding that DC produced IL-12 upon challenge indicated that DC were also capable of directing the type of Th-cell response [28–30], with IL-12 being the key cytokine in driving Th1 responses. The recognition that DC-derived cytokines were key in driving Th-cell responses was complemented by studies showing that the interaction of DC and Th was subject to modulation by multiple factors [31]. In this, the strength of stimulation as determined by the antigen dose and co-stimulation, genetic background, tissue-derived factors, and specific cytokines produced after encounter with pathogen-derived products, were shown to play an important role in determining whether a Th1 or Th2 response develops.

20.2.1
The Strength of DC–Th-cell Interaction

The strength of the interaction mediated through the T-cell receptor and the MHC/peptide complex and the dose of antigen were found to affect Th-cell development [31–34]. *In vitro* stimulation of OVA-peptide specific TCR transgenic $CD4^+$ T cells with low antigen doses presented by bone-marrow derived DC or splenic $CD11c^+$ DC induces an IL-4 response, whereas high antigen doses favors increased IFN-γ production [32–35]. Separation of splenic $CD11c^+$ DC into $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-$ DC showed that both DC populations had a similar effect of the antigen dose on Th-cell polarization [35]. The Th1-cell development at high antigen doses was only partially blocked by anti-IL-12p40 antibodies, suggesting the additional involvement of Th1-inducing factors other than IL-12 and IL-23. Similar to conventional DC, bone marrow-derived plasmacytoid DC, although less efficient in inducing T-cell proliferation at low antigen doses, showed the same flexible ability to drive the development of Th1 cells at high antigen dose, and preferentially Th2 cells at low antigen doses [35]. These findings clearly point out that intrinsically DC populations have the ability to direct both Th1 and Th2 development under specific circumstances. The enhanced Th1-cell development at high antigen doses can be explained, at least in part, by enhanced activation of T cells resulting in strong upregulation of CD40-ligand on Th cells, which delivers a positive feedback signal to DC to augment its IL-12p70 production [34]. When the CD40/CD40-ligand interaction was prevented using anti-CD40-ligand antibodies or DC from CD40 knock-out mice, Th1 polarization was inhibited at high antigen doses. Similar results were obtained using IL-12p35 knock-out mice [34]. Th2-cell development at low antigen doses was dependent on IL-4, since neutralization of IL-4 inhibited Th2-cell development [33]. However, DC obtained from IL-4 knock-out and wild-type mice both induced Th2 responses to a similar degree, which indicates that T cells, but not DC, are the source of IL-4 (Boonstra and O'Garra, unpublished). Additionally, it was reported that a strong TCR signal resulted in sustained Erk activation and Th1 differentiation, whereas a weak signal induces transient Erk activation and an increase in IL-4 production [36].

In agreement with the mouse studies, activated human monocyte-derived DC pulsed with high or low doses of toxic shock syndrome toxin-1 (TSST-1) stimulated autologous T cells at high TSST doses to develop Th1 responses, and at low doses to develop Th2 responses [37]. In MLR responses, although the absolute dose of antigen cannot be established, it was shown that human monocyte-derived DC promote Th2-cell differentiation at low DC:T cell ratio (1:300) in a primary MLR, and Th1-cell differentiation at a high DC:T ratio (1:4) [38]. These findings were reproduced using mouse splenic $CD11c^+$ DC in an antigen-specific system, with a low DC:T ratio resulting in a mixed Th0/Th2 phenotype, and a high ratio in the development of Th1 cells [39] (Boonstra and O'Garra, unpublished).

20.2.2
Co-stimulators

Enhanced contact of the T cell with the APC or a prolonged duration of the interaction can also be achieved by co-stimulatory molecules. Indeed, in addition to CD40/CD40ligand interaction, a role for CD28/B-7 [40], OX40/OX40-ligand [41], and LFA-1/ICAM [42, 43] has been shown in Th polarization. The results of this strengthened interaction between the APC and the T cells may lower the threshold (i.e. effective antigen dose) needed for CD40-ligand upregulation and Th1-cell development [44].

20.2.3
Genetic Background

The genetic background has been shown to influence the ability of DC to develop Th1 or Th2 responses. This has been shown elegantly by comparing the responses to *Leishmania major* infection in B10.D2 mice, which develop a protective Th1 response with self-healing lesions, and in BALB/c mice, which develop a Th2 response resulting in the development of progressive lesions [45]. Although $CD11c^{+}CD8\alpha^{-}$ DC from both mouse strains primed $CD4^{+}$ T cells *in vivo*, $CD11c^{+}CD8\alpha^{-}$ DC obtained from BALB/c mice stimulated T cells to produce less IFN-γ and more IL-5 as compared to $CD11c^{+}CD8\alpha^{-}$ DC from B10.D2 mice. No differences in cytokine expression, including IL-12, were found when comparing DC from both strains, except for higher IL-1β expression by DC isolated from B10.D2 mice, which was shown to reduce Th2-cell responses upon injection *in vivo* [45].

20.3
Opposing Concepts: Pre-programmed versus Flexible DC Direct Th-cell Development

The ability of DC to direct Th1 or Th2-cell responses is dependent on cytokines, the strength of the stimulus, as determined by the antigen dose and co-stimulators, as well the genetic background, resulting in a high degree of flexibility of DC, which enables DC to respond to a wide variety of stimuli appropriately. It came therefore as a surprise that in 1999 almost simultaneously, in both mouse and human studies, it was suggested that specific DC subpopulations were pre-programmed to induce the development of Th1 cells, and others to induce Th2 responses, and that they were fixed in doing so [46–48].

20.3.1
Mouse Dendritic Cell Populations in Directing Th-cell Development

In vivo it was shown that splenic $CD11c^{+}CD8\alpha^{+}$ DC, cultured overnight with GM–CSF and pulsed with KLH, induced the development of Th1 cells *in vivo*, whereas antigen-pulsed $CD11c^{+}CD8\alpha^{-}$ DC induced Th2 cells when injected into

mice [47]. Th1 development induced by the transfer of CD11c$^+$CD8α$^+$ DC was IL-12 mediated, since CD11c$^+$CD8α$^+$ DC isolated from IL-12p40 knock-out mice did not promote Th1-cell development. Furthermore, IL-12p70 production was higher by CD11c$^+$CD8α$^+$ DC as compared to CD11c$^+$CD8α$^-$ DC upon stimulation [47]. These findings led to the hypothesis that specific DC populations produced IL-12 and promoted Th1 responses, whereas other DC populations did not produce IL-12 and as a result favored the development of Th2 responses (Fig. 20.1). Similar findings were reported by Pulendran et al., who showed using Flt3-ligand-treated mice, that OVA-peptide pulsed CD11c$^+$CD8α$^-$ DC induced higher levels of IL-4 production by T cells, while similar levels of IFN-γ were induced by T cells upon transfer of CD11c$^+$CD8α$^-$ DC and CD11c$^+$CD8α$^+$ DC into mice [48].

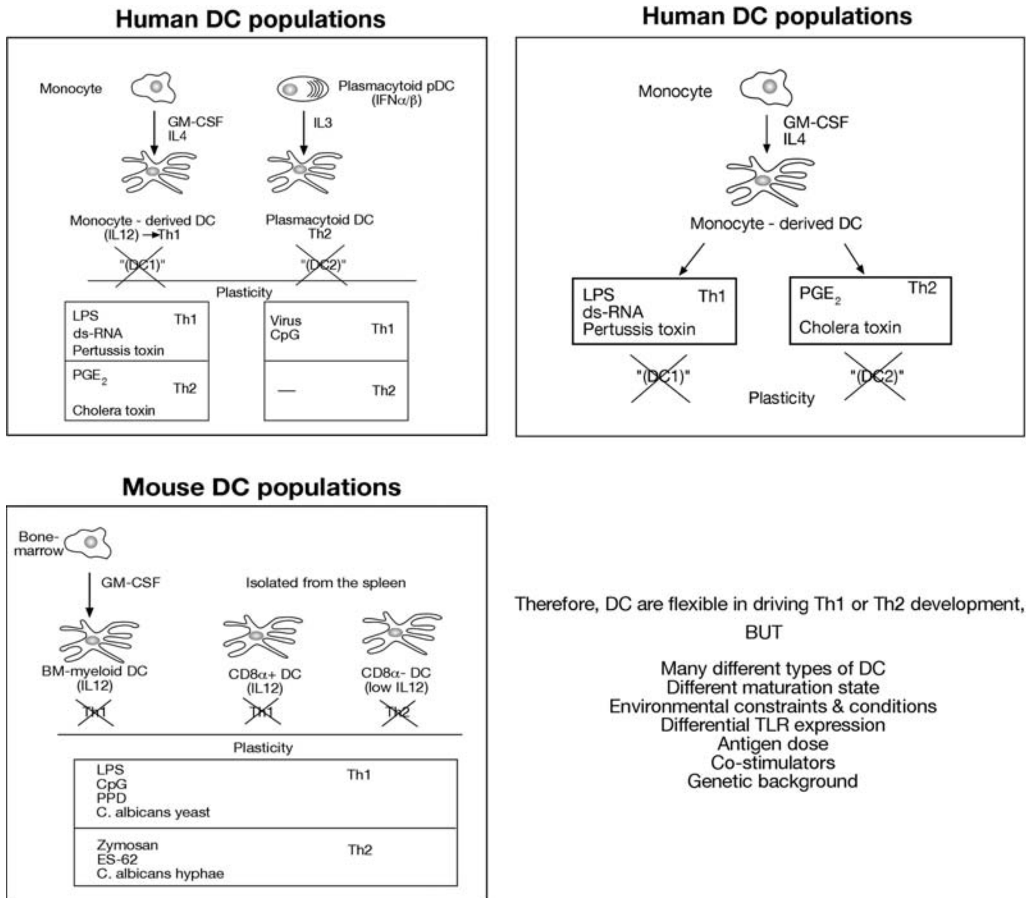

Fig. 20.1 Human and mouse DC populations have a high degree of flexibility in driving Th-cell regulation, but there are many variables: different types of dendritic cell; in different maturation states; environmental constraints and conditions; different TLR expression; antigen dose; co-stimulators and genetic background.

The proposed concept that specific DC populations were fixed in promoting Th-cell responses was soon after challenged by a number of studies (Fig. 20.1). *In vivo* transfer of peptide pulsed $CD8\alpha^+$ and $CD8\alpha^-$ DC, which were not cultured, but freshly isolated from the spleen, showed that both splenic DC subsets have the potential to prime Th1 and Th2 cells when injected intravenously [49]. Also, stimulation with different pathogenic products demonstrated the flexibility of DC populations in promoting Th1 or Th2 responses. *In vitro*, both sorted $CD8\alpha^+$ and $CD8\alpha^-$ DC stimulated with purified protein derivative (PPD) from *Mycobacterium tuberculosis* showed augmented development of IFN-γ producing cells, while both subsets responded to zymosan by enhanced priming of IL-4 producing cells [39]. Also mouse bone marrow derived myeloid DC stimulated with LPS or CpG DNA induced Th1 responses [35], whereas stimulation with a filarial nematode glycoprotein ES-62 favored the development of Th2 cells *in vitro* [50, 51]. Different forms of the fungus *Candida albicans* can instruct DC to induce either Th1 or Th2 responses. At the yeast stage, the fungus induced IL-12 production by a mouse DC cell line and promoted Th1 responses, while at the hyphae stage the fungus surprisingly stimulated these DC to produce IL-4 and induce Th2 responses [52]. However, using a wide range of stimuli, many groups have been unable to detect the production of IL-4 protein or expression of IL-4 mRNA by primary mouse DC [34, 51, 53].

Limited data is available on the ability of mouse plasmacytoid DC to direct Th-cell development. Freshly isolated plasmacytoid DC from the spleen express low levels of MHC II and co-stimulators and induce weak T-cell proliferation, and consequently could not trigger Th-cell development [35, 54]. However, stimulation of plasmacytoid DC with CpG DNA induced the expression of MHC II and costimulators, resulting in enhanced $CD4^+$ T-cell proliferation. Furthermore, strong Th1-cell development was observed upon activation of $CD4^+$ T cells with CpG-stimulated plasmacytoid DC, which was partly IL-12-dependent [35]. In contrast, Krug et al. were unable to induce Th1-cell development even upon stimulation of mouse plasmacytoid DC with CpG, and showed a role for plasmacytoid DC in memory responses [55]. However, as will be discussed below, the same group showed that human plasmacytoid DC induced strong Th1 responses when stimulated with influenza virus [56]. Clearly, these reports demonstrate the high degree of functional plasticity of mouse DC populations upon challenge with a variety of pathogens, or due to variations in their micro-environment.

20.3.2
Human Dendritic Cell Populations in Directing Th-cell Development

Also initial studies using human DC populations suggested that human DC were pre-programmed to induce the development of a particular Th phenotype. It was reported that human CD40-ligand-activated DC, differentiated from monocytes by culturing with GM–CSF and IL-4, were found to induce Th1-cell development *in vitro*, which was at least in part IL-12 dependent, whereas CD40-ligand-activated DC derived from blood $CD11c^-CD4^+CD3^-$ plasmacytoid cells induced Th2 cells [46]. CD40-ligand activated monocyte-derived DC produced IL-12, whereas the

ability to produce IL-12 of activated plasmacytoid DC, which secrete high levels of IFN-α, remains controversial [46]. Also these findings, suggesting that specific human DC are pre-programmed to induce either Th1 or Th2 cells, have been challenged, as will be discussed below. Consequently, the proposed nomenclature for monocyte-derived DC as DC1, and plasmacytoid DC as DC2, reflecting their suggested ability to be fixed in driving Th1 or Th2 responses, respectively, is confusing and is not preferred.

Cella et al. challenged the finding that human plasmacytoid DC were fixed in driving exclusively Th2 responses by showing that, when stimulated with either CD40-ligand or influenza virus, IL-3 cultured plasmacytoid DC induced the development of strong Th1 responses [56]. Simultaneously, Kadowaki et al., showed that IL-3 cultured plasmacytoid DC induce human allogeneic T cells to induce a Th2 cytokine profile, whereas herpes simplex virus activated plasmacytoid DC induced the development of Th1 cells, producing IFN-γ and IL-10 [57]. The ability of virus-activated plasmacytoid DC to induce IFN-γ production in T cells was dependent on IFN-α/β, but independent of IL-12, whereas both IL-12 and IFN-α/β were involved for CD40-ligand-activated plasmacytoid DC [56, 57]. The factors responsible for inducing Th2-cell development are unknown. However, addition of IL-12 to IL-3-cultured plasmacytoid DC co-cultures with T cells showed an enhanced percentage of IFN-γ producing cells, but no decreased frequency of IL-4 producing cells, suggesting that the absence of IL-12 does not lead to a Th2 default phenotype [57]. A role for OX40-ligand has been suggested in promoting Th2-cell development by plasmacytoid DC, since plasmacytoid DC cultured with IL-3 expressed high levels of OX40-ligand, and neutralization of OX40-ligand significantly inhibited the ability of IL-3 activated plasmacytoid DC to induce the development of Th2 cells [58].

Similar to plasmacytoid DC, human monocyte-derived DC also induce the differentiation into both Th1 and Th2 cells depending on the stimulus or presence of pro/anti-inflammatory molecules. Pathogen-derived products, such as lipopolysaccharide, double stranded RNA, and fixed *Staphylococcus aureus* Cowan's strain (SAC), as well as T-cell-derived factors, such as CD40-ligand and IFN-γ can all stimulate monocyte-derived DC to induce Th1 development, via IL-12 dependent mechanisms [30, 59, 60]. On the other hand, activation of monocyte-derived DC in the presence of *Schistosoma* egg antigen, cholera toxin, or anti-inflammatory molecules such as IL-10, TGF-β, prostaglandin-E_2 and steroids promotes Th2-cell development [61–64]. An early trigger of the allergic immune cascade might be represented by human thymic stromal lymphopoietin (TSLP), a novel IL-7 like cytokine that is produced by human epithelial, stromal, and mast cells, and in particular, is highly expressed by keratinocytes of atopic dermatitis patients, but not in other types of skin inflammation. TSLP activates human blood $CD11c^+$ DC, but not monocyte-derived DC, to produce Th2-attracting chemokines but no IL-12, and to induce naïve $CD4^+$ and $CD8^+$ T-cell differentiation into effector cells with a typical pro-allergic phenotype [65].

Thus, although DC are flexible, they may be pre-programmed to drive either Th1 or Th2-cell development in response to certain pathogen-derived products.

20.4
Differential TLR Expression by Distinct Dendritic Cell Populations

The Th1-inducing capacity of DC is largely determined by their production of IL-12p70 and/or IFN-α upon stimulation by pathogen-derived products [28, 37, 46, 47, 56, 60, 66–68]. A number of studies have suggested that distinct DC populations have an intrinsic inability to produce IL-12p70 and/or IFN-α, which consequently results in a differential ability to drive Th1 or Th2 cells [46–48]. However, this lack of production of IL-12 and/or IFN-α by specific DC subpopulations could be the result of the lack of expression of the corresponding Toll-like receptor (TLR) or other pathogen recognition receptor, or due to negative regulation of IL-12 or IFN-α expression.

In human and mice, distinct DC subsets have been shown to respond to different microbial products partly due to the differential expression of different TLR. Human monocyte-derived DC express TLR2, TLR3, TLR4, TLR8 and low levels of TLR7, and respond to their respective ligands [67, 69–71]. Similarly to human monocyte-derived DC, mouse bone-marrow derived myeloid DC express TLR2 and TLR4, and respond to their respective ligands Pam3Cys and LPS, whereas mouse splenic $CD11c^+CD8\alpha^+$, $CD11c^+CD8\alpha^-$ DC and plasmacytoid DC express little to no TLR2 and TLR4 and do not respond to their respective ligands. Both human and mouse plasmacytoid DC express TLR7 and TLR9, and respond to their ligands R-848 and CpG to produce IFN-α and IL-12 [35, 67, 72, 73]. However, it should be noted that mouse TLR9 expression is not restricted to plasmacytoid DC, but is expressed more broadly, albeit at lower levels, on other DC subsets [35]. The cytokine production induced by TLR ligation is partly determined by intrinsic properties of the specific DC population. As a consequence of TLR9 ligation, mouse plasmacytoid DC produce IL-12p70 as well as IFN-α, whereas bone marrow-derived myeloid DC produce only IL-12p70 [35]. Also, ligation of TLR7 using imiquimod or R-848 stimulation of human plasmacytoid and blood myeloid $CD11c^+$ DC induced the production of IFN-α and IL-12p70 respectively, both of which resulted in Th1-cell development [67]. This suggests that intrinsic differences between DC populations exist resulting in distinct cytokine profiles. Differential TLR expression may explain why some pathogen-derived products induce differential cytokine production in specific DC subtypes, and may thus have the potential under certain circumstances to direct Th1 or Th2 development.

20.4.1
Modulation of TLR Expression

At present, limited data is available on the regulation of TLR expression, and therefore it can not be excluded that under specific stimulation conditions, or during inflammation or pathology, TLR expression can be modulated, thereby changing the responsiveness to TLR ligation of specific DC subtypes. Some studies point out that this is indeed the case. Maturation of monocytes into monocyte-derived DC has been shown to result in enhanced TLR3 and TLR4 expression [74]. Stimulation

with LPS enhanced the expression of TLR9 by human PBMC [75] and mouse DC [76]. Also, cytokines can affect TLR expression. IL-10 was shown to prevent LPS-induced TLR4 mRNA upregulation in monoytes [74], and GM-CSF enhanced the expression of TLR2 and TLR9 by human neutrophils [77].

20.5 Modulation of IL-12p70 or IFN-α Production

Stimulation of DC with TLR ligands alone generally induces low levels of IL-12p70, and often induces only IL-12p40 production. Enhanced expression of the limiting IL-12p35 subunit can be achieved by additional stimulation with various cytokines or with T-cell associated signals. A synergistic signal for IL-12p70 production is delivered by direct cell–cell contact with activated T cells via the interaction of CD40L on T cells with CD40 on DC [29, 30, 78, 79]. Various studies have shown that $CD11c^+CD8\alpha^+$DC are superior over $CD11c^+CD8\alpha^-$ DC to produce IL-12p70 upon stimulation with pathogen-derived products alone [47, 80, 81]. However, under optimal stimulation conditions both splenic DC populations have the ability to produce significant IL-12p70 production. Following stimulation with CpG or soluble toxoplasma antigen (STAg) alone low amounts of IL-12p70 are produced by splenic $CD11c^+CD8\alpha^+$DC only, whereas both $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-$ DC produce IL-12p70 upon simultaneous microbial stimulation and CD40-ligation [79]. CD40-ligation is not only essential for IL-12p70, but also for IL-10 production [80] (Boonstra and O'Garra, unpublished), and augments the production of other cytokines, such as IFN-α, IL-12p40 and TNF (Boonstra and O'Garra, unpublished).

Further regulation of IL-12 production can be exerted by cytokines or other regulatory molecules present in the micro-milieu of the DC. Cytokines such as IFN-γ, GM-CSF and surprisingly also the Th2-associated cytokines IL-4 and IL-13, are potent enhancers of IL-12p70 production [68, 82–84]. These cytokines can be produced by activated T cells, but also NK cell derived IFN-γ plays an important role in promoting Th1-cell development [85–87]. On the other hand, suppression of IL-12 production has been described by IL-10, IFN-α, TGF-β, prostagladin-E_2, and steroids [88–92].

IL-10 has been shown to suppress IL-12p70 production by DC and macrophages, resulting in the inhibition of Th1-cell development [93, 94]. Both $CD8\alpha^+$ and $CD8\alpha^-$ DC obtained from IL-10 knock-out mice produce IL-12p70 *in vitro* upon stimulation with fixed *Staphylococcus aureus* and IFN-γ [53]. *In vivo,* infection of IL-10 knock-out mice with *Brucella abortus* resulted in higher IL-12p40 production by both splenic $CD8\alpha^+$ and $CD8\alpha^-$ DC as compared to wild type mice [95], clearly showing the ability of IL-10 to inhibit IL-12 production by DC. Recently, it was demonstrated that IL-4 enhances LPS or CpG induced IL-12p70 production by decreasing the production of IL-10 by DC. In IL-10-deficient mice the ability of IL-4 to upregulate IL-12p70 was not observed [96].

Besides IL-10, also IFN-α can inhibit the production of IL-12 [91, 92]. Both human and mouse plasmacytoid DC produce high levels of IFN-α in response to viruses, and upon stimulation with agonists of TLR9 (CpG DNA) and TLR7 (R848). The early production of IFN-β and IFN-α4 can promote the production of other IFN-α subtypes by positive feedback mechanisms. Dalod et al., showed that following infection with murine cytomegalovirus (MCMV), plasmacytoid DC ($CD11c^+GR1^+CD11b^-CD8\alpha^+$) were the major source of IFN-α/β and IL-12 [97]. Conventional splenic $CD11c^+CD11b^-CD8\alpha^+$ produced low levels of IL-12. However, blocking the production of IFN-α/β *in vivo* by depletion of plasmacytoid DC did not significantly affect IL-12 production in serum, which was shown to be due to increased IL-12 production by the conventional splenic $CD11c^+CD11b^-CD8\alpha^+$ DC. Similarly, abrogation of IFN-α/β signaling using MCMV infected IFN-α/βR knock out mice, resulted in increased IL-12 production by splenic $CD11c^+CD11b^-CD8\alpha^+$ DC [97]. These and other findings indicate that the major cell type responsible for IL-12 production can vary depending on the pathogen (plasmacytoid DC for MCMV, $CD11c^+CD8\alpha^+$ DC for STAg), and is highly regulated by cytokines in the micro-environment. Interestingly, the same study showed that high viral dose resulted in higher levels of IL-12, but lower levels of IFN-α as compared to a lower viral dose [97].

As outlined above, the levels of IL-12 and/or IFN-α produced by a specific DC population are determined by multiple factors, such as the nature of the pathogen, the additional requirement for T cell–derived signals, and the presence of cytokines that promote or suppress IL-12 production. Moreover, also the stage of maturation of DC populations is an important factor. IL-12p70 production by human monocyte-derived DC stimulated with LPS or CD40-ligand can be strongly enhanced by IFN-γ. However, maturation of monocyte-derived DC resulted in the gradual loss of responsiveness to IFN-γ due to loss of the IFN-γR [60]. Similarly, as discussed above, DC populations at different stages of their maturation may vary in responsiveness to pathogens due to regulation of TLR expression.

It has been recently shown that ligands for different TLR strongly synergize in inducing IL-12p70 production by either human or mouse DC [98]. In particular, ligands for TLR utilizing the adaptor molecules MyD88 and TRIF can when combined induce up to two orders of magnitude more IL-12p70 than when used alone [98]. The synergism is evident particularly at the level of accumulation of transcripts of IL-12p35 that requires an autocrine effect of secreted type I IFN signaling through STAT-1. Indeed, type I IFN, which at high doses inhibits IL-12p40 production, may increase, similarly to but less potently than IFN-γ, IL-12p70 production by mouse [98] and human DC [99]. Thus, pathogens able to simultaneously trigger different TLR may be able to produce an early burst of biologically active IL-12p70 in a cell-autonomous way with the help of autocrine type I IFN. Maintenance and amplification of IL-12 production then requires the participation of IFN-γ or IL-4 produced by innate effector cells (NK, NKT, mast cells), or by antigen-specific T cells as well as co-stimulatory molecules such as CD40-ligand expressed by T cells activated in an antigen-specific or non-specific way.

Additionally, activation of DC by LPS resulted in a transient IL-12 production, followed by a refractory period to further stimulation [37]. More specifically, mono-

cyte-derived DC stimulated with LPS for 8 h showed augmented IL-12p70 production when restimulated via CD40-ligation, whereas microbial stimulation 24 h prior to CD40-ligation did not result in detectable IL-12p70 production. As a consequence, soon after activation, DC induce Th1-cell development upon encounter of T cells, whereas the interaction with T cells at later time points preferentially promotes Th0 or Th2 responses. The inability to produced IL-12 at later time points after activation has been referred to as "exhaustion" [37] or "paralysis" [100] of IL-12 production.

The tight regulation of IL-12p70 and IFN-α/β production by DC, which is dictated by the type of pathogen, the involvement of T-cell feedback, and cytokines offers the DC a high degree of flexibility to promote Th1 or Th2 polarization.

20.6 Factors Responsible for Driving Th2-cell Development

The factors responsible for Th2-cell polarization by DC are still largely unclear. Although, IL-4 is a key factor in driving Th2 responses, there is still debate whether DC can produce IL-4. On the basis of early findings that mouse splenic CD8α^- DC do not produce IL-12 and promote Th2 responses, and reports that "Th2-stimuli" induce little IL-12 production, the hypothesis was put forward that Th2 cells develop by default in the absence of IL-12. However, recent studies showed that in the absence of IL-12, Th1-inducing pathogens did not induce Th2 responses by default [101, 102]. Using IL-12 knock-out mice on both BALB/c and C57Bl/6 genetic backgrounds, mice infected with *Mycobacterium avium* or primed with STAg from *Toxoplasma gondii* exhibited a marked reduction in *in vitro* IFN-γ production, but no significant IL-4 production in the absence of IL-12 [102]. The development of Th2 responses by stimulation of mouse splenic DC with *Schistosoma* egg antigen (SEA) correlated with unaltered or downregulation of the expression of co-stimulatory molecules and chemokines upon stimulation, and a temporary delay in T-cell cycling [103]. In humans, TSLP, produced by epithelial and stromal cells, is very potent in endowing CD11c$^+$ blood DC and tonsil DC with the ability to produce Th2-attracting chemokines and to induce Th2 responses [65].

20.7 Modulation by Tissue Factors

In the mouse, CD11c$^+$CD8α^+ and CD11c$^+$CD8α^- DC reside in distinct locations in the spleen in steady-state conditions: CD11c$^+$CD8α^+ DC reside in the T-cell areas, whereas CD11c$^+$CD8α^- DC are located mainly in the marginal zone. Therefore, these DC populations are exposed to a different micro-milieu with different cells and cytokines. CD11c$^+$CD8α^+ DC are surrounded by lymphocytes, and are therefore likely under inflammatory conditions to be exposed to T-cell-derived cyto-

kines, such as IFN-γ. $CD11c^{+}CD8\alpha^{-}$ DC are surrounded by marginal zone macrophages, which may, under specific conditions produce more IL-10, prostaglandin-E_2 and pro-inflammatory cytokines such as TNF. Therefore, under steady state conditions, the findings that splenic $CD11c^{+}CD8\alpha^{+}$ and $CD11c^{+}CD8\alpha^{-}$ DC have a differential ability to produce cytokines [47, 80, 81] may be the results of the effect of exposure to a different cytokine repertoire *in vivo*. These conditions may change during immune responses when $CD11c^{+}CD8\alpha^{-}$ DC migrate from the marginal zone to the T-cell area of the spleen [104].

A role for the tissue environment in modulating Th1 and Th2 responses has been most extensively studied in mice. In the Peyer's patches, $CD11c^{+}CD8\alpha^{-}$ DC produce IL-10 and little IL-12p70 upon stimulation with trimeric CD40-ligand or SAC and IFN-γ. Besides IFN-γ, these cells induced T cells to produce IL-4 and IL-10, giving it a mixed Th1/Th2 phenotype [105]. As a comparison, splenic $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC induced T cells to produce similar levels of IFN-γ as compared to Peyer's patches DC but low IL-4 and IL-10.

Also comparison of DC isolated from the liver and the spleen, showed functional differences with respect to IL-12p70 production, which were ascribed to the distinct anatomical location. In the spleen, CD40-ligand activated splenic $CD8\alpha^{+}$ DC produce high levels of IL-12p70 as compared to $CD8\alpha^{-}$ DC. However, CD40-ligand activated liver $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC both induce high IL-12p70 levels, which were comparable to the levels produced by splenic $CD8\alpha^{+}$ DC [106]. These findings suggest that there is no intrinsic difference between $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC, but that the observed differences are due to distinct anatomical location of the DC with different cytokine environments. The function of respiratory tract DC isolated from rat has been shown to be suppressed by pulmonary alveolar macrophages. *In vivo* depletion of alveolar macrophages enhanced the MHC II expression and T-cell stimulatory capacity of DC [107]. Additionally, respiratory tract DC preferentially induce Th2 responses when pulsed *in vitro* and transferred *in vivo* [108]. In this context, it has recently been shown that depletion of DC, from the airways of CD11c-diphteria toxin receptor transgenic mice during ovalbumin aerosol challenge, abolished the characteristics of asthma, including eosinophilic inflammation, bronchial hyper-reactivity, and the production of Th2 cytokines (IL-4, IL-5, IL-13). Reconstitution of CD11c-depleted mice with $CD11c^{+}$ DC restored the asthmatic symptoms [109]. Culture of rat respiratory tract DC *in vitro* in the presence of GM–CSF resulted in the production of Th1 cytokines [108].

20.8 Concluding Remarks

Exposure of DC to various stimuli during inflammation dramatically modulates its environment, and consequently the characteristics of the DC itself.

The ability of the antigen dose to affect Th-cell development could provide an explanation why different DC subsets have been suggested to intrinsically direct Th1

or Th2-cell development [46–48]. It is plausible that due to possible differences in their stage of maturation, they express different levels of MHC II and/or co-stimulatory molecules, and therefore may have different effective antigen doses presented by the APC to the T cell. In addition, DC subpopulations at different stages of their maturation may process antigens with different efficacies and consequently present different amounts of MHC II/peptide on their surface, which affects the effective antigen dose and consequently to altered Th-cell development.

The net result of the complex interplay of the state of maturation of the DC, previous encounters, tissue factors, the effects of pathogen-derived products, the strength of signal as well as genetic factors makes the DC extremely efficient as a sensor of diverse pathogenic challenges, and highly flexible in directing the appropriate immune responses. On the other hand, the ability to differentially express TLR, and thus respond to different TLR ligands, may suggest that certain DC may be destined to respond to distinct pathogen-derived products, and thus different pathogens, to direct Th1-cell development. These issues remain to be determined, and are fundamental for vaccination strategies and therapeutic interventions.

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Microbial Instruction of Dendritic Cells

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21.1
Introduction

Efficient protection against the different types of microbe that may invade our body requires tailor-made responses by natural and antigen-specific immune cells. Natural defense is mediated by stromal cells of body-lining tissues, such as epithelial cells, fibroblast and endothelial cells, as well as more specialized migratory immune cells such as NK cells, granulocytes, macrophages and dendritic cells (DC). Importantly, these epithelial DC cells are the unique natural immune cells that inititiate adaptive, antigen-specific responses mediated by T cells and, eventually, T cell-dependent B cells [1]. DC initiate antigen-specific immunity by stimulating naïve T cells with microbial antigen (signal 1). In addition, DC decide between immunity and tolerance by expressing variable patterns of T-cell co-stimulatory molecules (signal 2). Finally, DC orchestrate protective immune responses through the expression variable sets of T-cell-polarizing molecules (signal 3) that promote the development of certain classes of effector T helper cells (Th1, Th2 or regulatory T cells) [2]. In this chapter we will review the current knowledge on how DC determine the class of the antigen-specific immune response through their ability to recognize different pathogen types.

21.2
Effector Th1 and Th2 Cells and Regulatory T Cells

Polarized effector Th1 and Th2 cells develop from a common pool of naïve precursor T cells upon stimulation by DC. Th1 cells produce high levels of IFN-γ and TNF-β, cytokines that are instrumental in the induction of protective cell-mediated immune responses against intracellular pathogens such as viruses, certain types of (myco)bacteria and protozoa. Effector Th2 cells produce high levels of IL4, IL-5 and IL-13 and are involved in protection against helminths [3, 4]. Recently, a third subset of T cells has been described, the regulatory T cells. These T cells play a major

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role in the maintenance of tolerance against self and harmless environmental proteins [5]. In addition, most pathogens induce variable amounts of regulatory T cells that hinder the development of effector cells and protective immunity, thereby contributing to the success of the pathogen. Paradoxically, these cells are also beneficial to the host. They prevent excessive damage as a result of full blown effector T cell-driven inflammation and ensure the development of solid memory as a result of the longer persistence of the pathogen [6, 7]. Two subsets of Treg can be distinguished: naturally occurring $CD4^+CD25^+$ regulatory T cells [8], which are primarily involved in central and peripheral tolerance to auto-antigens [9], and adaptive regulatory T cells [5], such as Tr1 cells and Th3 cells [10, 11]. Adaptive regulatory T cells originate, like Th1 and Th2 cells, from uncommitted peripheral naïve or central memory Th cells upon activation by antigen in a certain immunological context [12] and normally respond to innocuous foreign antigens and are primarily associated with mucosal tolerance to ubiquitous antigens and nonpathogenic microflora [13, 14]. The outcome of an immune response to a certain pathogen is thus determined by the balanced development of effector and/or regulatory T cells, which is orchestrated by DC.

21.3
Dendritic Cells and Pattern Recognition Receptors

Two major types of DC are classic CD11c+ myeloid DC and $CD123^+$ (IL-3Rα) plasmacytoid DC (PDC) [15, 16]. Immature myeloid DC continuously patrol the body-lining epithelia for incoming pathogens. Upon pathogen recognition, they undergo a process of maturation, including their migration toward the draining lymphoid tissues and their acquisition of potent T-cell stimulatory capacity [17, 18]. Like all natural immune cells, DC detect pathogens with germ-line encoded “pattern recognition receptors” (PPRs), that recognize “pathogen-associated molecular patterns” (PAMPs), which are evolutionary conserved microbial molecules essential to pathogen function [19, 20]. An important group of PRR are the family of Toll-like receptors (TLR). To date, eleven different human TLR (TLR1–11) have been described [21].The classical human myeloid $CD11c^+$ DC express TLR1-6 and TLR8 [22, 23], which allow them to recognize a broad panel of bacterial and viral compounds, as discussed in detail below.

PDC are found in immune tissues and in variable numbers in peripheral tissues, in particular during inflammation. They produce high levels of type I interferons (IFN) upon viral infection and are believed to play an important role in antiviral defense as natural immune cells [24]. The TLR expression profiles of PDC strongly differ from that of myeloid DC [22, 23]. In contrast to the wide distribution of TLR on myeloid DC, PDC selectively express intracellular TLR7 and TLR9, recognizing single stranded (ss)RNA [25, 26] and CpG-containing DNA motifs [27], respectively. At present, the role of PDC in the initiation of specific immunity is not entirely clear, but they are very likely to play a role in the activation of effector T cells at the site of infection.

TLR ligation of myeloid DC leads to their activation and immediately results in the production of pro-inflammatory cytokines, which contribute to local innate immune responses In addition, TLR signaling initiates the program of DC maturation, leading to the transformation of immature DC into potent effector DC that are uniquely capable of initiating specific immune responses by driving the development of effector Th cells from naïve Th cells [17, 28]. Amongst others, these mature DC express selective sets of T cell-polarizing molecules, either soluble or membrane-bound, that determine the balance between Th1, Th2 or regulatory T-cell development [2]. The expression profile of these T cell-polarizing molecules by DC is dependent on and imprinted by the binding of pathogen to selective PRRs (e.g. TLR) of DC in their immature state, resulting in selective programming of these DC during their maturation. Basically, DC exposed to intracellular pathogens express T-cell-polarizing molecules promoting Th1 responses, whereas certain helminths prime DC for the expression of molecules that drive the development of Th2 cells (Fig. 21.1) [29]. Furthermore, certain types of pathogens prime for DC with a strong capability to induce the development of regulatory T cells [30].

Another large family of PRRs is formed by the C-type lectins that recognize specific carbohydrate structures present in pathogens, such as mannosylated lipoarabinomannan (ManLAM) on mycobacteria [31, 32]. In contrast to ligation of TLRs, the binding of a C-type lectins results in the internalization of bound pathogens followed processing for antigen presentation [33]. Although *in vitro*-generated human immature monocyte-derived DC express many C-type lectins, only a few have been identified on myeloid blood DC [15]. In contrast, plamacytoid DC express

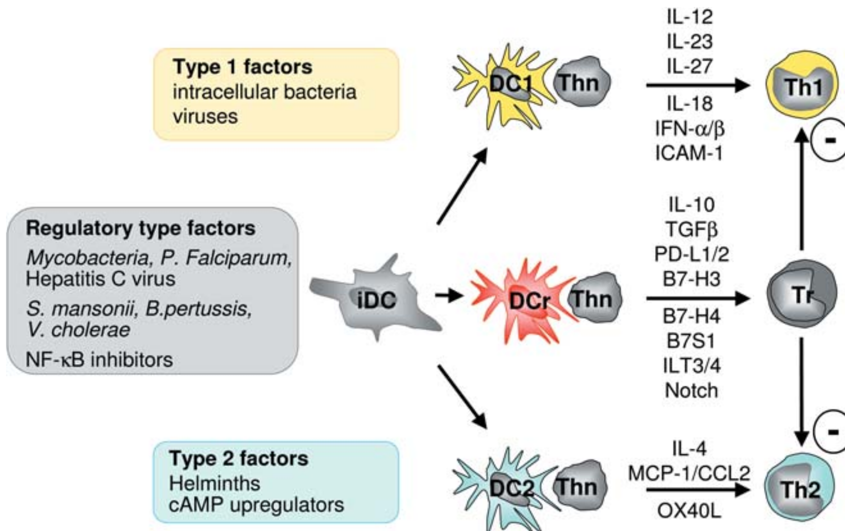

Fig. 21.1 Dendritic cell polarization. Schematic representation of factors that influence the T-cell polarizing capacity of DC and the expression of molecules (either cytokines or membrane-bound molecules) that drive the development of either Th1, Th2 or adaptive regulatory T cells. (iDC immature DC).

Blood DC Antigen (BDCA)-2, Dectin-1 and DEC205. Most C-type lectins on DC are type II transmembrane proteins containing at least one carbohydrate recognition domain which bears, depending on the amino acid sequence, specificity for mannose, galactose or fructose structures [34].

Little is known about the role of C-type lectins in the priming of DC for the induction Th1 or Th2 cells. Recognition of pathogens by C-type lectins will favor immune suppression whereas concomitant TLR triggering overrules the tolerizing function of C-type lectins, resulting in immune activation [35]. The role of C-type lectins in pathogen recognition will be discussed in more detail in Chapter 7.

Apart from these two major groups of PRRs, a number of other molecules expressed on antigen presenting cells, have been identified to bind pathogen-derived compounds, although information about the consequences for the Th-cell development is scarce. The type 3 complement receptor (CR3) is capable of binding *Mycobacterium tuberculosis*, yeast-derived zymosan and filamentous hemagglutinin (FHA) from *Bordetella pertussis* [36, 37]. Also different types of scavenger receptors are known to recognize pathogens such as *Neisseria meningitidis* via SR-A [38] and *Plasmodium falciparum* [39]. Also the Nod proteins (Nod1 and Nod2) have recently been shown to be intracellular PRRs that bacterial peptidoglycan (PGN) although Nod1 and Nod2 detect distinct motifs within this structure [40]. Interestingly, a mutation in *Nod2* resulting in the loss of the capacity to bind PGN-derived muramyl dipeptide, is associated with Crohn's disease [41, 42].

21.4 DC-derived Factors that Promote Th1, Th2 or Regulatory T-cell Responses

21.4.1 Th1 Cell-promoting Factors

Of all known Th1-promoting factors, IL-12 has been studied most extensively. The bioactive IL-12p70 molecule is a heterodimer composed of a 35-kDa light chain (p35) and a covalently bound 40-kDa heavy chain (p40). The p40 subunit is produced in large excess, but cannot induce signal transduction upon receptor ligation. Homodimers of IL-12p40 may even prevent IL-12 p70 signaling [43]. The IL-12 receptor (IL-12R) is primarily expressed on NK cells and activated T cells and consists of two chains, the IL-12Rβ1 and the signaling IL-12Rβ2 chain. IL-12p40 binds the IL-12Rβ1 chain, whereas the p35 subunit ligates the IL-12Rβ2 chain [44]. Recently, two related heterodimeric cytokines have been identified, IL-23 and IL-27, which together with IL-12 are referred to as the IL-12 family. Like IL-12, these two cytokines are mainly produced by hematopoietic phagocytic cells (monocytes and macrophages) and DC and their production is enhanced by IFN-γ [4]. IL-23 consists of the IL-12p40 subunit and a unique p19 subunit. The IL-23 receptor consists of the IL-12Rβ1 chain that binds p40, whereas p19 binds to the IL-23R, a novel gp130-like chain [45]. IL-27 is a heterodimer of p28 and the Epstein–Barr virus-

induced gene 3 (EBI3), an α-receptor-like soluble chain homologous to IL-12p40 [46, 47]. In contrast to EBI3, which, like p40, is produced in large excess, the subunit p28 cannot be secreted without EBI3 [47]. EBI3 binds to a receptor chain designated T-cell cytokine receptor (TCCR) or WSX-1 and p28 ligates the gp130 chain [47, 48].

Although all three members of the IL-12 family induce the production of IFN-γ by activated T cells and NK cells, their effects on memory versus naïve Th cells and on the magnitude of the response may be different. Naïve Th cells rapidly produce IFN-γ in response to IL-12 or IL-27 and develop into Th1 cells [47, 43]. However, the effects of IL-27 are dependent on IL-12 or IL-18 [49]. Unlike IL-12, IL-23 does not have a potent effect on naïve Th cells but is particularly efficient in promoting the production of IFN-γ in memory Th cells [50]. Expression ratios of these IFN-γ-inducing cytokines depend upon distinct ligation of PRRs by pathogenic compounds as will be discussed below.

Another group of cytokines that may play a role in the development of Th1 cells is the family of type I IFNs which consists of the homologous cytokines IFN-α, β, τ and ω. These cytokines have important antiviral activities, but have also been shown to possess a wide range of immunoregulatory functions [51]. IFN-α is a subfamily of various homologous IFN species that differ in their antiviral effects and their effects on T-cell proliferation and NK cell activity [51]. Although it is widely accepted that type I IFNs drive Th1 development, the evidence is not substantial. Several studies have demonstrated that they increase IFN-γ mRNA and protein production in naïve and previously activated Th cells, but this increase is by far not as high as the increase induced by IL-12 [9]. Part of the effect of IFN-α on IFN-γ production may be explained indirectly by the transient upregulation of the IL-12Rβ2, resulting in an enhanced sensitivity for IL-12 or by inhibition of IL-4 production [52].

Not only the IL-12 family cytokines but also other factors may play a role in the induction of Th1 cells. IL-18 was first described as IFN-γ-inducing factor and was found to be circulating in mice during endotoxemia [53]. It is constitutively expressed as pro-IL-18 by monocytes, macrophages and DC, both in mice and humans. The production of biologically active IL-18 is induced by factors (such as lipopolysaccharide (LPS) or gram-positive bacteria) that stimulate the expression of IL-1β-converting enzyme that cleaves proIL-18 into mature IL-18 [54]. In contrast to IL-12, IL-18 by itself induces only low levels of IFN-γ in human $CD4^+$ T cells. However, in the additional presence of IL-12, IL-18 strongly enhances IFN-γ production [55] and IL-18 itself does not induce the development of Th1 cells from naïve precursors [56].

Finally, not only soluble factors but also membrane-bound molecules may promote Th1 responses. One such molecule is ICAM-1 that ligates LFA-1 on the T cells. ICAM-1/LFA-1 interaction supports adhesion between DC and T cells. Both in mice and human ICAM-1/LFA-1 interaction promotes the induction of Th1 responses, which is particularly evident in the absence of T cell-polarizing cytokines, such as IL-4 or IL-12 [57–59].

21.4.2 Th2 Cell-promoting Factors

In contrast to the abundant knowledge about Th1-inducing factors, little is known about the active induction of Th2 cells by DC. It is widely accepted that IL-4 is a potent inducer of Th2 cells from naïve precursors and that the main source of IL-4 is the Th-cell population itself, indicating that IL-4 acts as an autocrine factor amplifying IL-4 production by developing Th2 cells. Some reports suggest that murine DC may have the capacity to produce low levels of IL-4 in response to certain yeast hyphae or virus species but no reports on human DC-derived IL-4 are available [60, 61]. An important prerequisite for the induction of Th2 responses is that the capacity of DC to produce IL-12-family members is down-regulated, to prevent induction of IFN-γ production. Indeed, IL-12 is capable of restoring the IL-12-responsiveness in established effector Th2 cells *in vitro*, resulting in high levels of IFN-γ and down-regulated IL-4 production [62]. Although it was suggested earlier that Th2 responses are the mere result of the absence of IL-12 (or IL-12 family members) [63], we [64] and others [65, 66] have clearly shown that the development of Th2 cells driven by helminth-primed DC requires active Th2 cell-polarizing factors expressed by these DC.

A molecule expressed both by murine and human DC that is clearly involved in the induction of Th2 cells from naïve precursors is membrane-bound OX40L, the ligand of OX40 (CD134) expressed on T cells. OX40L-OX40 interaction not only co-stimulates proliferation of $CD4^+$ T cells, but was also proposed to contribute to Th2 development. Co-stimulation of murine or human naïve Th cells with an OX40L-transfected cell line enhances the expression of IL-4 and promotes the development of Th2 cells *in vitro* [67, 68].

21.4.3 Regulatory T-cell-promoting Factors

In all of the studies on the induction of regulatory T cells by DC, IL-10 has been implicated as the (co-)responsible factor. IL-10 is a pleiotropic cytokine that functions at different levels of the immune response. For example, it blocks proliferation and cytokine production by T cells, by inhibiting the phosphorylation of CD28, thereby abrogating downstream signaling [69]. *In vitro* studies showed that naïve $CD4^+$ T cells that are cultured in IL-10 become regulatory T cells with the capacity to suppress the activation and proliferation of bystander T cells [70]. IL-10 may also induce tolerance indirectly, as it inhibits the full maturation of DC and downregulates their MHC class II and IL-12 expression [71, 72].

Apart from IL-10, several other factors expressed by DC may be involved in the induction of regulatory T cells, including TGF-β, novel inhibitory members of the B7 family, members of the Notch signaling pathway (e.g. Serrate1) [73, 74] or ILT3 and ILT4 [75, 76]. The expanding family of B7 molecules are all expressed by DC and other APC. They are ligands for co-stimulatory molecules on T cells and their

expression is probably tightly regulated as they play critical roles in the control and fine-tuning of the immune response [77]. The best-characterized B7 family members are the ligands of CD28 and CTLA-4, CD80 and CD86, also known as B7.1 and B7.2. Both are upregulated on DC upon activation [20].

More recently discovered family members are the two counter-structures of programmed death1 (PD-1), i.e. the broadly expressed PD-L1 (also called B7-H1) [78] and the DC-specific PD-L2 (also called B7-DC) [79]. Yet others are B7-H3 and B7-H4. B7-H3 (also referred to as B7-RP2) binds a still unknown receptor on T cells that is distinct from CD28, CTLA-4, ICOS and PD-1 [80]. Experiments with mice deficient for either of these B7 family molecules or experiments using blocking reagents suggested negative regulatory functions in the immune response [81–85]. Although it is tempting to speculate on a role of these molecules in the induction of regulatory T cells, it can not be excluded that loss of tolerance observed in these mouse models is merely due to T-cell anergy or apoptosis.

21.5 TLR-mediated Activation of DC by Microbes and their Compounds

Accumulating data underline the concept that ligation of different PRR during microbial exposure primes for functional maturation of effector DC with distinct Th-polarizing capacities. The original belief was that TLR triggering by definition always results in the development of Th1 cells. However, as discussed below, it is now clear that differences in the expression of T-cell polarizing factors may be a reflection of TLR ligation.

21.5.1 TLR2

TLR2 can form heterodimers with TLR1 or with TLR6 and thus distinguish the subtle differences between triacyllipopeptides of gram-negative bacteria (recognized by TLR1/2), and diacyllipopeptides of mycoplasma (recognized by TLR2/6) [86, 87]. Triggering of these different TLR2-containing heterodimers translates into different cytokine patterns. Ligation of TLR1/2 by lipoproteins from *Mycobacterium* species results in low IL-12 but enhanced IL-23 production [88]. An additional nonredundant role IL-27 is suggested by a study of *M. bovis* bacillus Calmette–Guerin infection in IL-27R-deficient ($WSX^{-/-}$) mice, showing impaired early IFN-γ production and poorly differentiated granulomas, despite the availability of IL-12 and IL-23 [89].

TLR2/6 ligation, on the other hand, leads to other cytokine profiles. Apart from mycoplasmal diacyllipopeptides, this heterodimer recognizes zymosan from yeast cell walls and PGN from gram-positive bacteria [90, 91]. However, none of these compounds induces IL-12 production, but they lead to the production of IL-10, instead [62, 88, 92, 93]. Data on the consequence of TLR2/6 ligation for T-cell-pola-

rization varies considerably between different studies. Indeed, DC activated with mycoplasmal lipopeptide 2 were reported to induce unpolarized T-cell responses whereas zymosan primed for Th2-promoting DC [92, 93] and PGN for Th1-promoting DC with an increased expression of IL-23-p19 [62]. How these findings relate to the above mentioned TLR2/6-mediated IL-10 production remains unclear. Clearly, ligation of TLR2 by schistosomal-derived lyso-phosphatidylserine (lyso-PS) induces IL-10 production, as well. In this case, TLR2 ligation leads to the development of DC that promote the development of regulatory T cells from naïve precursors in a IL-10-dependent fashion [94].

21.5.2
TLR3

TLR3 recognizes dsRNA derived from viruses as well as the eggs of the helminth *Schistosoma mansonii* [95, 96] and promotes cross-priming to virus-infected cells [97]. Virus-derived dsRNA primes *in vitro* for a human DC phenotype that strongly supports Th1 cell development without enhanced IL-12 production [64]. *In vitro* studies with human monocyte-derived DC primed with dsRNA suggest the alternative involvement of IL-27 (A.J. van Beelen, unpublished results), type I IFN [98] and/or ICAM-1 [59] in the induction of Th1 cells. IL-12-deficient mice are able to clear viral infections, also suggesting a role for other Th1-promoting factors. It should be noted, however, that dsRNA can bypass TLR3 by binding to the intracellular receptor protein kinase R (PKR), which induces high levels of IFN-α in murine CD11^{+} DC [99].

21.5.3
TLR4

A well-studied PAMP/TLR-interaction is the binding of LPS from Gram negative bacteria to TLR4 [100]. Ligation of TLR4 by LPS induces low levels IL-12, IL-23 and IL-27 in monocyte-derived DC. Consequently, LPS-primed DC do induce Th1 cell responses, but clearly not as potently as dsRNA-primed DC [64, 98, 101]. Indeed, the Th1-driving capacity of LPS-primed DC is readily overruled by the presence of exogenous Th-cell-polarizing factors such as IFN-γ or PGE2 that potently enhance or decrease the IL-12 production [102]. LPS derived from distinct Gram negative bacteria may induce different levels of Th1- or Th2-promoting factors. For example, LPS derived from *Escherichia coli* binds TLR4, induces IL-12 production and primes for Th1 responses whereas LPS from *Prophyomonas gingivalis*, which may not ligate TLR4, fails to induce IL-12 and primes for Th2 cell responses [103]. These findings may possibly be due to differences in TLR4 requirements. Very recently, it was demonstrated that the phosphorylcholine-containing glycoprotein from the filarial nematode *Acanthocheilonem vitae*, ES-62, binds to TLR4 and signals via Myd88 [104]. This molecule has anti-inflammatory capacities and has been described to induce both Th2 as well as regulatory T cells [105].

21.5.4 TLR5

Only one ligand has been described for TLR5, i.e. the bacterial flagellin, a major component of bacterial flagella [106]. Whether TLR5-mediated activation of DC promotes a certain type of Th-cell response, remains to be established. One study using human monocyte-derived DC *in vitro* showed that flagellin and LPS induced similar levels of IL-12 and promoted Th1 responses to the same extend [107], whereas others showed that flagellin-activated human DC did not produce IL-12p70 [108]. This latter observation is corroborated by a murine *in vivo* study with flagellin from *Salmonella typhimurium*, demonstrating that flagellin induces the production of IL-12p40, but not the biological active IL-12p70, and induces Th2 responses [109]. However, the response to flagellin may be dependent on the context of presentation, as the soluble flagellar protein FliC alone induces a Th2 response in mice *in vivo*, whereas the response to FliC on intact *Salmonella* results in a Th1 response [110].

21.5.5 TLR7/8

Although TLR7 and TLR8 largely recognize the same structures, the expression pattern of these closely related intracellular TLR is quite distinct. In humans, TLR7 is strongly expressed by PDC, but only weakly by $CD11c^+$ blood DC, and not at all by monocyte-derived DC. TLR8, in contrast, is expressed both by monocyte-derived DC and $CD11c^+$ DC, but not by PDC. [22, 23, 111]. TLR7 and TLR8 recognize antiviral imidazolquinoline peptides and synthetic structures like R848 and loxorubine [107, 112, 113]. More recently it was discovered that viral ssRNA is the physiological ligand of TLR7 and TLR8. Murine PDC produce IFN-α and IL-12 in response to ssRNA, whereas macrophages and DC produce only IL-12 in these conditions [25, 26]. Although it is not yet known whether DC activated by ssRNA will indeed induce a Th1 cell response, ligation of TLR7 and TLR8 by imidazolquinolines, both in mice and man, modulates antigen-specific Th2 responses into Th1 cell responses [114, 115] which could be inhibited by the addition of a neutralizing anti-IL-12 antibody [114].

21.5.6 TLR9

TLR9 in humans is only expressed on PDC, whereas in mice it is expressed both by $CD11c^+$ DC and by PDC [101, 116]. TLR9 recognizes unmethylated CpG-containing bacterial DNA [27]. Activation of PDC with such CpG motifs results in the strong expression of IFN-α and promotion of Th1 cells [22, 23]. The production of IL-12 by human PDC *in vivo* is controversial [111, 117], but PDC precultured in IL-3 and subsequently stimulated with TLR9-binding CpG motifs in the additional

presence of CD40L are efficient producers of IL-12p70 [118]. IL-3, like the ligation of BDCA-2, inhibits the production of type I IFN and allows for the production of IL-12p70 [119]. In contrast to human PDC, murine PDC readily produce IL-12p70 in response to various viruses and TLR ligands [120].

21.5.7 TLR10/11

TLR10 is expressed in the lung and by B cells. Its ligand(s) and function are as yet unknown, although a genetic variation in TLR10 is associated with the risk of developing asthma [121]. TLR11 is widely distributed in mice, including expression by macrophages, liver cells, kidney cells and bladder epithelial cells. It recognizes a structure expressed by uropathogenic bacteria and mice lacking TLR11 are highly susceptible to infection with these bacteria [122]. As for TLR10, a role for TLR11 in T-cell polarization has not been reported. A human equivalent of TLR11 has not yet been described.

21.6 Th1 Cell-promoting DC

Many pathogenic compounds prime DC for the induction of Th1 cells, as has been described above, although the expression of the various Th1-promoting factors may differ. IL-12 is probably the best-studied Th1-promoting factor. Its production is readily induced by stimulation of immature DC by microbial compounds. However, only certain intracellular pathogens prime immature DC to develop into high-IL-12-producing mature DC. These high levels of IL-12 are produced upon CD40 ligation by the rapidly induced CD40L on activated naïve T cells in the draining lymph nodes. This indicates that the intrinsic capacity of mature effector DC to produce high levels of IL-12 (or other polarizing factors) is imprinted by previous exposure to pathogens during their immature phase in the infected tissues. Only the later CD40-CD40L interaction allows for full expression of the polarizing signals by mature DC and consequently for the selective development of Th1 or Th2 responses.

Examples of pathogens that indeed prime for high levels of IL-12 production upon subsequent CD40 ligation, are *Toxoplasma gondii, Leishmania major and Mycobacteria* species [123, 124]. The importance of IL-12 for the protection against these intracellular pathogens is corroborated by observations in patients with mutations in *IL-12p40* or *IL-12Rβ1* and, therefore, do not produce or respond to IL-12 and IL-23. Many of these patients develop chronic infections with *Mycobacteria* species and *Salmonella*. They can, however, efficiently clear viral and extracellular infections [125, 126]. Similarly, IL-12p35-deficient mice still develop polarized Th1 responses to certain mycobacterial infections, provided the p40 subunits are intact, suggesting a pronounced role of IL-23 [127, 128].

21.7 Th2 Cell-promoting DC

Several parasitic helminths as well as the hyphae from of the yeast *Candida albicans* have been reported to prime DC for the promotion of Th2 cells [60, 64–66]. Recently data are immerging about the PRRs involved in the recognition of helminths or helminth-derived structures. As has been pointed out previously several helminth-derived products may activate TLR2, TLR3 or TLR 4 [96, 104, 129] and drive the development of Th2 cells. But also sugar-containing structures, possibly binding to C-type lectins, are involved in the induction of a Th2-promoting phenotype in DC [130]. The role of C-type lectins in the induction of a Th2-promoting DC phenotype has been demonstrated in a recent study Bergman and coworkers [131] showing that a Lewis antigen positive variant of *Helicobacter pylori* escapes protective immunity by the binding to DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN), which blocks the induction of protective Th1 cells, whereas the Lewis antigen negative variant induces a potent protective Th1 cell response.

Little is known about the DC-expressed factors involved in the priming for Th2 cells. Only the expression of OX40L is clearly instrumental in the development of Th2 cells [64]. Resting murine and human DC do not express OX40L, but this is rapidly induced upon CD40 ligation. However, human effector monocyte-derived DC express differential levels of OX40L following CD40 ligation, depending on their priming conditions. Th1-inducing DC, primed with IFN-γ or poly I:C, do not express OX40L, whereas Th2-promoting DC primed with PGE_2 or schistosomal egg antigen (SEA) did express detectable levels of OX40L, which were further upregulated by subsequent CD40 ligation [64]. In addition, a critical role for OX40L-OX40 interaction in a functional Th2 response has been shown *in vivo* in a murine model of experimental leishmaniasis [132].

Interestingly, Th2-promoting DC with enhanced expression of OX40L show a low expression of ICAM-1 expression, whereas Th1-promoting DC show high levels of ICAM-1 and no OX40L [59, 64]. This may indicate possible opposing regulation of expression of these two co-stimulatory molecules.

21.8 Regulatory T-cell-promoting DC

It is now clear that DC not only play a role in the induction of effector Th1 or Th2 cells but can also promote the development of regulatory T cells that have the capacity to downregulate proliferation and cytokine production of effector Th1 or Th2 cells [133]. Paradoxically, pathogens that require Th1 or Th2 effector T cells to be successfully eliminated may actually induce immune suppression via the induction or expansion of regulatory T cells. This immune tolerance clearly benefits both the pathogen, by increasing its survival rate, and the host limiting the detrimental effects of chronic Th1 or Th2 responses [6, 134]. Pathogens may sustain immune tolerance via different mechanisms. Some pathogens, such as *P. falciparum*, hepa-

titis C virus and *Mycobacteria* species, prevent the maturation of DC into cells with a potent T-cell stimulatory capacity and, instead, lead to the development of tolerogenic DC [31, 135, 136]. *P. falciparum* infects erythrocytes which bind the scavenger receptor CD36 on DC which in turn inhibits LPS-induced maturation and, as a result, the T-cell stimulatory capacity of DC [135, 137]. Activation of murine DC with *Plasmodium*-infected eythrocytes induces the secretion of yet unidentified soluble inhibitory factors. In case of hepatitis C, virus-specific structures such as the core protein and nonstructural protein 3 inhibit the maturation, cytokine production and T-cell stimulatory capacity of DC, possibly resulting in the generation of the IL-10-producing $CD4^+$ T cells that have been shown to persist in patients infected with hepatitis C virus [138–140]. Yet another suppressive mechanism is the ligation of the C-type lectin DC-SIGN by ManLAM derived from *Mycobacteria* species. This interaction has been demonstrated to inhibit the LPS-induced maturation and IL-12 production by DC, but enhances their IL-10 production [141]. Whole mycobacteria do inhibit IL-12 production via DC-SIGN but, in contrast to ManLAM, do not inhibit DC maturation, suggesting that other compounds of mycobacteria overrule the inhibition of maturation [142].

Other pathogens allow the full maturation of DC but still induce the development of regulatory T cells. One of the first reported examples of this category of pathogens is *Schistosoma mansonii*. *S. mansonii*-derived lyso-PS ligates TLR2 and drives the development of regulatory DC that promote the generation of regulatory T cells. Lyso-PS does not inhibit LPS-induced maturation, but inhibits IL-12 production while enhancing the production of IL-10. This DC-derived IL-10 which contributes together with a yet unknown membrane-bound factor contributes to the development of regulatory T cells [94]. In addition, injection of the *Schistosoma egg*-derived glycoconjugate LNFPIII in mice induced the presence of an APC population that produced high levels of IL-10 and TGFβ and efficiently suppressed the proliferation of T cells *in vitro* [143]. Indeed, in patients suffering from chronic helminth infection antigen-specific regulatory T cells could be isolated and where characterized by the secretion of elevated levels of IL-10 and/or TGFβ and are associated with immunosuppression [144, 145].

Also *B. pertussis* induces the development of regulatory DC without inhibiting their maturation. The responsible virulence factor, filamentous haemaglutinin (FHA) inhibits IL-12 production but induces IL-10 production in murine DC and drives regulatory DC that promote the development of regulatory T cells capable of blocking secondary unrelated infections, e.g. with influenza virus [134]. Similar modulation of DC function has been described for another compound of *B. pertussis*, adenylate cyclase toxin, and for cholera toxin from *Vibrio cholerae* [146, 147].

21.9
Indirect Priming of DC

During primary infections, pathogens will not only affect DC directly but also indirectly via infected tissue cells. Various tissue cell types express TLR, recognize

pathogens and produce inflammatory mediators that can contribute to the shaping of the immune response. These factors include pro- and anti-inflammatory cytokines, chemokines, eicosanoids, heat-shock proteins and cell surface-bound molecules. We have shown that poly I:C-activated human primary keratinocytes express several TLRs including TLR3 and prime monocyte-derived DC for promoting Th1 responses which is dependent on TNF-α, IL-18 and IFN-α [148]. In the mouse, however, two independent studies showed that murine DC induce the development memory T-cell responses only if they are directly activated via their TLR, but not if they are activated indirectly by inflammatory signals from bystander DC [149, 150]. So far, there is no clear consensus on whether or not indirect activation of DC contributes to the formation of polarized Th1 or Th2 responses.

21.10
Concluding Remarks

The increasing knowledge of the biology of DC indicates both their complexity and their flexibility. It is clear that DC have a crucial role in properly adapting the class of immune response to the type of invading pathogen through the differential expression of T-cell polarizing signals upon ligation of particular PRRs. However, details on the underlying mechanisms are still largely unknown. It is becoming increasingly evident that pronounced Th1 responses are initiated upon ligation of the intracellular TLRs (i.e. TLR3, 7, 8 and 9) resulting in high expression of IL-12 family members. Also data have recently emerged on the mechanism underlying DC-driven Th2 cell development, for example in response to helminth infections. Currently, the generation of regulatory DC and the mechanisms by which they promote the development of regulatory T cells are major research topics in many laboratories, hopefully providing important new information in the near future.

The importance of the knowledge on how different T-cell responses evolve under the influence of differentially primed DC is that it may be helpful in the design of new therapies for a variety of immune disorders.

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VIII
CTL Priming and Crosspresentation

22
Crossprocessing and Crosspresentation

Mojca Škoberne and Nina Bhardwaj

22.1
Introduction

$CD8^+$ T cells are of prime importance for the recognition and elimination of tumor cells and cells that are infected by viruses or intracellular bacteria. They recognize short 8–10 AA residues bound to MHC class I molecules expressed on the surface of the majority of nucleated cells. MHC class I molecules display myriads of peptides that arise following the entry of cell-derived proteins into the endogenous (classical) antigen processing pathway. The initial activation of $CD8^+$ T cells is dependent upon professional antigen presenting cells (APCs) such as dendritic cells (DCs). APCs acquire antigens in the periphery and travel to secondary lymphoid organs where they stimulate T cells. However, as certain infectious agents may not target APCs, or APCs may rapidly succumb to infection, alternative mechanisms must be in place to induce protective $CD8^+$ T-cell responses. The same applies to antitumor immune responses where tumor cells by themselves are poorly functional as APCs.

It is now well established that APCs can internalize parts of- or whole infected cells and crosspresent the encoded antigens to T cells, a phenomenon termed "crosspriming", first coined in 1976 by Michael Bevan [1, 2]. Bevan injected mice with minor histocompatibility mismatched cells and observed that these cells prime host MHC I restricted cytotoxic T lymphocyte (CTL) responses to the exogenous antigen. These experiments were the first of several to establish the importance of crosspresentation in the induction of immune responses to viral and tumor antigens, as well as tolerance to self antigens.

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22.2
Acquisition of Antigens for Crosspresentation

22.2.1
Cells that Crosspresent

The identity of the cells that crosspresent antigens is a matter of long and ongoing debate. Several cell types have been shown to crosspresent antigens including endothelial cells, B cells, macrophages and DCs, however only selected cells are involved in crosspriming *in vivo* as we discuss below [3–10].

Initial studies by Bevan et al. showed that T cells recognize minor histocompatibility antigens presented on the surface of splenocytes [1]. In the early 1990s Rock et al. showed that in spleens only certain cells can process and present extracellular antigens in the context of MHC class I molecules. These cells express MHC class II molecules and Fc receptors [11] and correspond to a macrophage/dendritic cell population. In the following years, as DCs were further characterized and their phagocytic capacity was confirmed [12, 13], their role in crosspresentation became more convincing. Shen et al. [14] addressed the dispute of whether DCs or contaminating macrophages in the DC cultures are responsible for crosspresentation by transducing GM-CSF into bone marrow cell cultures that were later immortalized. These cells expressed many DC characteristics and most importantly, they were able to present exogenous antigens to CTLs.

In more definitive studies, Kurts et al. [15] generated mice that selectively expressed MHC I molecules only in DCs, along with the ovalbumin (OVA) antigen under control of the rat insulin promoter (RIP), thereby restricting OVA expression to nonlymphoid tissue. When mice were injected with OVA-specific OT-I T cells, the T cells underwent substantial expansion in lymph nodes. These experiments indicated that DCs acquired OVA exogenously from the periphery, and crosspresented it to T cells within draining lymph nodes.

Ultimate confirmation of the primary role of DCs in crosspresentation (at least for the cell-associated antigens) came from Jung et al. [16]. The authors constructed a system where murine DCs were selectively rendered susceptible to killing by diptheria toxin. This approach enabled inducible *in vivo* depletion of DCs. Mice were adoptively transferred with OVA-specific OT-I cells, DCs were depleted and mice were injected with MHC I deficient splenocytes, loaded with OVA by osmotic shock. In such experimental conditions, but not in control mice where DCs were not depleted, stimulation of OT-I cells was greatly impaired.

In mice, however, DCs are not a uniform cell population. Based on the expression of CD8α, CD11b, CD4 and Gr-1, at least 6 different subsets have been described to date [17, 18]. The presentation of antigens by different DC subsets in mice seems complex and dependent on antigen form and route of inoculation. Soluble antigens injected intravenously (i.v.) can be presented to $CD4^+$ T cells by $CD8^-$ DCs but only $CD8^+$ DCs can crosspresent it to $CD8^+$ T cells [19]. OVA-immune complexes (ICs) on the other hand can be presented to $CD8^+$ T cells by both, $CD8^+$ and $CD8^-$ DCs. However, only the $CD8^-$ DCs crosspresent ICs to $CD4^+$ T cells [20].

The loss of all three Fc receptors impairs the presentation to $CD8^+$ T cells by $CD8^-$ DCs but not by $CD8^+$ DCs^- perhaps because these DCs may acquire ICs via complement fixation and/or they use a mechanism similar to uptake of soluble antigen [20]. Crosspresentation of cell-associated antigens is also dependent upon distinct DC subsets. When β2m deficient OVA-loaded splenocytes were injected into mice, only the $CD8^+$ subset of DCs was capable of crosspresenting the antigen to $CD8^+$ T cells [21]. The DCs of the $CD8\alpha^+$ subset are superior in comparison to the ones of $CD8\alpha^-$ subsets in internalization of dead cells *in vitro* and *in vivo* [22, 23], but not latex or bacteria [22]. The mechanistic basis for this is not known, however. While the $CD8\alpha^+$ DCs express slightly higher levels of apoptotic cell receptor CD36, the receptor appears to be dispensable for crosspresentation [24, 25].

The role of different DC subsets in cross-tolerance is equally complex. Initially it was shown that a bone marrow-derived cells were responsible for crosspresentation of pancreatic islet cell-associated OVA, and subsequent deletion of self-reactive $CD8^+$ T cells [26]. In the non-obese diabetic mice (NOD), diabetes is mediated through destruction of β islet cells by $CD4^+$ and $CD8^+$ T cells. When apoptosis of islet cells was deliberately induced, the $CD11b^+$ DC subset was shown to be responsible for increased presentation of the β islet antigens in draining lymph nodes. The treatment resulted in decreased $CD4^+$ T-cell responses to islet cells, development of regulatory T cells and ultimately, protection of mice to diabetes [27]. Belz et al. showed that expression of OVA or glycoprotein B under RIP, led to their presentation by $CD8\alpha^+$ $CD11b^-$ DCs and deletional tolerance of $CD8^+$ T cells [28]. In a different model, gastric parietal cell-specific H^+/K^+-ATPase that is recognized by autoreactive T cells was constitutively processed by both CD8α high and CD8α low gastric DCs and transported to draining gastric lymph nodes [29]. Of note, the route of antigen inoculation can influence which DC subtype acquires antigens. Chung et al. demonstrated that intravenously injected OVA was preferentially internalized by $CD8\alpha^+$ DCs, whereas intragastric injection of the protein resulted in uptake by $CD8\alpha^-CD11b^+$ DCs [30].

The identity of the crosspresenting cell is further complicated by observations that both immunity and tolerance may be induced after one DC subset migrates from the periphery and transfers the antigen to a second, lymph-node resident DC subset [31, 32].

How these differential roles of various mice DC subsets relate to humans is not known to date. In humans only two major DC subsets have been identified so far: myeloid (mDC) and plasmacytoid (pDC) DCs. Human mDCs were shown to efficiently take up and present apoptotic and necrotic tissues [33, 34] and to be involved in priming as well as tolerization [35–41]. On the contrary, human pDCs are primarily known as professional interferon α (IFNα) producing cells (IPC), and associated with innate antiviral responses. Recently, their role in adaptive immune responses is becoming better recognized [42–44], although their ability to crosspresent antigens is poorly explored. Studies indicate, that compared to mDCs they are inferior in the uptake of cellular material and may express different sets of antigen degrading enzymes such as cathepsins [44–47]. A recent study by Schnurr et al. showed that while $CD1c^+$ DCs efficiently crosspresent epitopes of full length NY-

ESO 1 protein in the context of class I MHC molecules, pDCs could only present MHC class II restricted epitopes [48]. Future studies will be needed to draw the final parallels between human and murine DC subtypes and to define their roles in the crosspresentation of antigens.

22.2.2 Sources of Antigens and Receptors involved in Crosspresentation

Phagocytes may acquire antigens for crosspresentation by macropinocytosis, phagocytosis or receptor-mediated endocytosis, however not all of them are equally efficient [49]. Sources of antigens that enter the crosspresentation pathway are equally diverse and include soluble antigens, immune complexes, antigens coupled to latex beads, heat-shock protein (HSP) bound peptides, cell-associated antigens, exosomes or even synthetic structures such as microspheres [50, 51]. (Antigen sources and receptors involved in their recognition are summarized in Table 22.1).

Below we discuss the relevance and mechanisms underlying the uptake of several of the above.

22.2.2.1 Apoptotic Cells

Apoptosis is a physiological form of cell death during embryogenesis, tissue turnover and following infection. Apoptotic cells are probably one of the most notorious "natural" sources of antigens for crosspresentation. The antigens packed within the cells result in crosspresentation that is several hundred-fold more efficient in comparison to soluble antigens [49], presumably because apoptotic cells are recognized by macrophages and DC via an array of receptors [52]. These include type I and type II integrins, phosphatidyl serine receptor (PSR), scavenger receptor CD36 and Lox-1, to name only a few. Macrophages and DCs however may not play equivalent roles in the uptake of apoptotic cells. Macrophages primarily function as apoptotic cell scavengers and thus prevent their secondary necrosis, release of dying cell contents and inflammation that could lead to induction of autoimmune responses [53–58]. On the contrary, DCs are less efficient in clearing the apoptotic cells [33, 59] but as shown *in vitro* and *in vivo*, they process and present the antigens encoded within apoptotic cells to lymphocytes and may do so following ligation of chosen apoptotic cell receptors. Initial studies in this field come from Albert et al. [34], who showed that virus-infected apoptotic cells are an excellent source of antigen for crosspresentation by DCs. DCs utilize scavenger receptor CD36 and two αv-integrin receptors (αvβ3 and αvβ5) to endocytose the apoptotic cells [33, 60]. However, the αv-integrins and CD36 were subsequently shown to be dispensable for crosspresentation, at least in mice [24, 25], indicating a redundancy amongst receptors in apoptotic cell phagocytosis. Indeed, Lox-1, PSR, complement receptors 3 and 4 and FcR were subsequently shown not only to be expressed on DCs, but also to bind apoptotic cells (e.g. after opsonization with iC3b or antibodies). Interestingly, phagocytes use these receptors not only to dock and internalize the apoptotic cells but also to localize the apoptotic cells. Phagocytic cells reach sites of exten-

Tab. 22.1 Selected sources of antigens for crosspresentation and their receptors.

	Receptors involved in recognition	*Reference*
Apoptotic cells	CD36 (may via TSP-1)	24, 25, 33
	αvβ5	24, 33
	αvβ3	24
	SR-A	170
	Lox-1	171
	PSR	172, 173
	MER	174
	CD14	175
	CD91	176
	CR3	41, 68, 69, 177
	CR4	41, 68, 69, 177
	ABC	178
	CD31	179
	G2A	63
Necrotic cells	Lox-1	171
(and components	CD91	86
encoded within)	TLR 3 (RNA)	180
	TLR 9 (DNA)	181
	TLR 2 (HSPs, HMGB-1)	182–185
	TLR 4 (HSPs, HMGB-1)	182–185
	RAGE (HMGB-1)	186
Exosomes	αvβ3	117
	CD11a	117
	CD54	117
Nibbling of live cells	SR-A	108
Immune complexes	Fcγ receptors	20
	CR3, CR4 ?	20
	nonspecific uptake similar to soluble antigens	20
Heat shock proteins	CD91	86, 176, 187, 188
	CD14	189, 190
	CD40	93, 191, 192
	CD36	193
	Lox-1	171
	TLR2	183–185
	TLR4	183–185
	SR-A	194

sive apoptotic cell death by chemotaxis to lysophosphatidyl choline (LPC) [61], a molecule exposed on the surface of apoptotic cells in a PLA2-dependent manner [62] and then released by apoptotic cells as a chemoattractant. LPC is recognized by G2A, a recently identified receptor on phagocytes [63].

Uptake of infected apoptotic cells by DCs initiates effector T-cell responses. In contrast, the crosspresentation of uninfected counterparts in steady-state condi-

tions may result in cross-tolerance [23, 27, 64]. Dying cells provide an excellent source of self antigens and can be carried to draining lymph nodes in the absence of inflammation [29, 65] presumably by DCs [29]. Uninfected apoptotic cells generally do not induce DC maturation [41, 66–69] and can even interfere with the response of APCs to inflammatory stimuli [41, 52]. Therefore, upon apoptotic cell uptake, DCs remain immature. Immature DCs have been shown to induce regulatory T cells – either *in vitro,* by repetitive stimulation of naïve T cells by allogenic immature DCs [70] or *in vivo,* following injection of immature DCs loaded with influenza antigen that induced regulatory $CD8^+$ T cells in immunized individuals [35, 36]. Taken together, this has led to the hypothesis that uptake of apoptotic cells in the absence of inflammation will tolerize the responding T cells. Such a point of view has gained support through several recent publications [26, 27, 41, 66–69, 71, 72].

Mechanistically, the inhibitory effects of apoptotic cells are most likely linked to stimulation of discrete receptors. Binding of CD36, CD51 or CR3 by antibodies or apoptotic cells prevented DC maturation upon exposure to inflammatory signals such as LPS or CD40L [41, 66–69, 73, 74]. Until recently, it was not known whether modulation occurs at the transcriptional or posttranscriptional level, nor what the primary molecular targets are. Cvetanovic et al. [75] showed that binding of apoptotic cells to macrophages modulates NFκB and AP-1-dependent gene transcription. The authors suggest that a common transcriptional co-activator, such as CBP or its paralog p300, could be the target of apoptotic modulation [75], but experimental confirmation is still pending.

22.2.2.2 **Necrotic Cells**

Necrosis is a physical disruption of cells that occurs mostly in pathological situations or may follow apoptotic cell death when cells are not removed efficiently. In contrast to apoptotic cells where antigens remain confined in membrane-bound form, necrotic cells release their contents which are not only a source of antigens for crosspresentation [76–78], but simultaneously provide endogenous factors that induce activation of DCs [79–84]. Stimulatory properties of cell lysates are primarily attributed to various factors for example endogenous HSPs [85, 86], HMGB-1 or uric acid crystals [84, 87], to name a few. Given that antigens and maturation signals are delivered simultaneously it is not surprising that necrotic cells can elicit protective virus and tumor-specific T-cell responses [77, 78]. The relative role and contribution of apoptotic versus necrotic cells towards crosspresentation by DCs remains controversial and the issue is complicated by the use of various antigens (tumor versus infectious) and model systems.

22.2.2.3 **Heat-shock Proteins**

HSPs are highly conserved and abundant chaperones. They represent up to 5% of total intracellular proteins and upon exposure to stress, their levels can reach as high as 15% [88]. Aside from a number of housekeeping functions, HSPs assist in

chaperoning non-covalently bound peptide antigens from the endosome or cytosol to MHC molecules [83]. When released into the extracellular environment, HSP-peptide complexes are recognized by APC by a myriad of receptors including CD91, CD14, CD40, CD36, Lox-1, TLR 2 and 4. HSPs induce APC activation or maturation [88, 89] and simultaneously deliver bound antigens for crosspresentation to T cells [86]. HSPs are recognized by DCs and channeled via receptor mediated endocytosis to the endocytic compartment [90]. A fraction of HSP-peptide complexes escapes destruction in phago-lysosomes, enters the cytosol and is processed in a TAP and proteasome-dependent manner [90].

Recognition of the stimulatory role of HSPs in the immune system can be attributed to the pioneering work of Srivastava [88] and Matzinger, who proposed the "danger hypothesis" [79]. To date numerous HSPs including gp96, HSP60, HSP70 and HSP90 have been associated with the induction of immune responses in mice and in humans [88]. Cytoplasmic HSP 70 and 90 and ER gp96 derived from virus-infected cells or tumor cells can be crosspresented, prime $CD8^+$ T cells and even induce protective immunity *in vivo* [77, 78, 91, 92]. Further support for their immunogenicity comes from the autoimmunity field. Millar et al. used a transgenic mouse model, where LCMV-GP is expressed in pancreatic islet cells, under the control of RIP. Normally, when the immunodominant epitope gp33 is administered to mice it tolerizes the responding T cells. However, when gp33 was co-administered with HSP70, the epitope specific T cells underwent autoimmune activation rather than anergy [93].

Shen et al. recently addressed the physiological relevance of HSPs in crosspresentation compared to other sources of antigens [94]. They used fibroblasts stably expressing OVA constructs in either a membrane bound, secreted or cytosolic form. After cell lysis, they exposed APCs to the three fractions and evaluated their ability to crossprime cytotoxic T lymphocytes to membrane-bound and cytosolic OVA. Whereas the cytosolic fraction could also include HSP-associated antigens the authors ascribe the primary role to native proteins. By depleting the cytosolic fraction of OVA with specific antibody, they abrogated the priming capacity of the cytosolic extract. The immune complexed OVA did not contain HSP70, HSP90, and grp94, suggesting that native protein vs HSP-associated peptides were a major source of antigen for crosspresentation. However, the contribution of other forms of HSPs cannot be formally excluded.

22.2.2.4 **Immune Complexes**

Fc receptors (FcRs) are a family of membrane glycoproteins that recognize and bind Fc portion of antibodies and include FcγR I (CD64), FcγR II (CD32) and FcγR III (CD64). Usually, the binding affinity and signaling potential of FcRs is greatly enchanced upon their oligomerization, thus in circumstances when immune complexes (ICs) of antibodies and antigens are present [95, 96]. FcRs are expressed on variety of hematopoietic cells, including mouse and human DCs. In mice, ICs are recognized by $CD8^+$ and $CD8^-$ DC subsets [20] and in humans immature DCs efficiently take up ICs [97, 98]. Among human DC subtypes cancer/testis antigen NY-

ESO-1 ICs were efficiently presented by monocyte-derived but not by $CD34^+$ DCs [99] and efficacy of presentation was maturation-dependent. Accordingly, Langerhans cells, that are sensitive to maturation by IFN-γ, acquired crosspresentation capacity only upon stimulation with the cytokine [100]. Endocytosis of ICs via FcRs is followed by delivery to cytosol and entry into endogenous antigen processing and presentation pathway [101]. Formation of immune complexes and recognition by FcRs is associated with increase in crosspresentation of antigens [102, 103]. FcRs not only provide antigen for crosspresentation but may also deliver a DC activation signal in certain circumstances [98, 102]. However, because mice and humans express activatory as well as inhibitory FcR isoforms, the final outcome is dependent on cumulative signals provided through the two [104]. In the latter study, tumor cells opsonized by antibodies were more efficiently crosspresented when the inhibitory FcR was blocked. The delivery of ICs to APCs appears to have a significant role *in vivo*, resulting in the crosspriming of tumor specific T-cell responses [105, 106].

22.2.2.5 **Nibbling from Live Cells**

Although cell death is a very efficient mechanism for delivery into crosspresentation pathway it is not always required. Several reports show that DCs and macrophages can acquire the antigens by "nibbling" from live cells [10, 107, 108]. The process involves membrane transfer from donor to acceptor cell in a receptor-dependent manner, involving at least scavenger receptor A [108]. Next to nibbling, transfer of peptides via gap junctions is another mechanism of antigen transfer among living cells as we describe later [109].

22.2.2.6 **Exosomes**

Exsosomes are small vesicles (50–100 nm) of endocytic origin that are formed by reverse budding of late endosome membrane [110]. Exosome formation was initially attributed to neoplastic cells but was then extended to a variety of hematopietic cells, including DCs [111–115]. Although the function of exosomes is not yet fully explored they might be a means of intercellular communication.

Exosomes express MHC molecules on their surface and when DC-derived exosomes were pulsed with peptides they could elicit effector T-cell responses in tumor bearing mice [115]. A similar protective effect was seen when DCs were pulsed with microbial antigens [116]. Later on it was shown that tumors themselves constitutively secrete exosomes that are loaded with tumor antigens. When such exosomes are taken up by DCs they elicited T-cell responses through crosspresentation [112]. Interestingly, when the uptake of exosomes was studied *in vivo*, it was noted that in mouse blood-derived exosomes are taken up primarily by immature $CD11c^+$ DCs, MOMA-1^+ and ER-$TR9^+$ macrophages. DCs internalized exosomes by means of αvβ3, CD11a and CD54 receptors in a process that did not induce their maturation [117]. Taken together, this suggests that exosomes can behave similarly to apoptotic cells. It is not inconceivable, therefore, that, depending

on the conditions, exosomes could shift priming to effector or tolerogenic responses, however experimental support for this is absent.

22.2.2.7 **TLR and MyD88 involvement in Crosspresentation**

The importance of TLR signaling in crosspresentation was first suggested from observations that mice injected with conjugates of TLR ligands and antigens, could make efficient CTL responses [118, 119]. Moreover, the development of these responses occurred in the absence of CD4 T-cell help [119]. Interestingly, using bone-marrow derived DCs, it was shown that only selected TLR agonists potentiated the crosspresentation of OVA protein by DCs, namely TLR3 and TLR9 ligands, poly I:C and CpG DNA, respectively [120]. This could partially be explained by observations from West et al, who showed that triggering of TLRs initially mobilizes the actin cytoskeleton, stimulates macropinocytosis and thus enhances the presentation of antigens in the context of MHC class I and II molecules [121]. However, the authors observed similar effect with several TLR ligands, including, CpG, poly I:C, LPS and PGN [121].

Involvement of TLRs is even more intriguing in the case of apoptotic cells. Apoptotic cells are involved in the induction of tolerance, and in the settting of infection, the stimulation of effector T-cell responses. The nature and sequence of events that shifts the balance to either tolerance or immunity has long been speculated. Schulz et al. in their elegant studies offer a rather simple explanation [122]. Using a model system consisting of apoptotic cells loaded with TLR3 ligand (dsRNA), they showed initially that ligation of TLR3 is critical for initiating crosspriming. To verify that this applies during actual virus infection, they make use of Semiliki Forest Virus (SFV) which has very limited ability to infect the CD8α^+ DCs in vitro, but can efficiently infect Vero cells. Vero cells were infected with genetically engineered OVA-expressing SFV that can undergo only a single round of infection (to prevent the possibility of indirect DC infection), induced to undergo apoptosis, and offered to DCs as a source of antigen. Only wild type but not TLR3$^{-/-}$ DCs were able to prime T-cell effectors *in vitro*. Similarly, *in vivo* priming to SFV-OVA was impaired in TLR3$^{-/-}$ mice, although not completely blocked. The effect could be mimicked by using OVA/poly I:C loaded Vero cells [122]. Activation of TLR3 stimulates DC maturation and secretion of variety of pro-inflammatory cytokines including type I IFNs that are known to enhance crosspriming [123]. The evaluation of crosspriming in TLR/IFNα/βR double KO mice will be necessary to define the role of type I IFNs in this model.

Chen et al. also used SFV-OVA virus and showed that directly infected DCs presented virus-encoded antigens to CD8$^+$ T cells in a MyD88-independent manner, whereas crosspresentation and OT-I OVA-specific CD8$^+$ T-cell stimulation was MyD88-dependent [124]. MyD88 is a signal adaptor molecule that is shared by most TLRs [125] and disruption of this molecule diminishes the expression of NFκB-dependant genes that participate in DC maturation. As TLR3 also signals in a MyD88-independent manner, participation of other MyD88-associated receptors in crosspriming to this virus can not be formally excluded. dsRNA can also trigger

DC maturation and type I IFN production through MyD88-independent, PKR-dependent or -independent mechanisms [125]. Thus, viruses which have dsRNA intermediates and which induce apoptosis, could promote crosspresentation by DCs through a number of mechanisms.

It will be interesting to see how dependence on TLRs and MyD88 correlates with observations published by Blander et al. [126] who show that phagosome maturation is regulated by the nature of its cargo. They studied uptake of bacteria and apoptotic cells by macrophages. Interestingly, bacteria but not apoptotic cells were rapidly localized in phago-lysosomes in a TLR and MyD88-dependent manner. When bacteria and apoptotic cells were incubated with macrophages simultaneously, co-localization of the two was not observed. Not surprisingly, and in support of observations made previously by Lucas et al. that simple addition of TLR agonists cannot modulate signals induced by apoptotic cells [127], the addition of LPS to apoptotic cells did not influence the rate of phago-lysosomes formation. The physiological relevance of this biased routing is unclear. Perhaps rapid phago-lysosomal formation after ingestion of bacteria ensures their rapid destruction while in the case of apoptotic cells the formation of late endosomes is delayed in order to allow antigenic peptides to enter the crosspresentation pathway. Trombetta et al., have shown recently that DCs express low levels of proteases in their phagosomes, but once stimulated to mature they up regulate expression and processing of internalized antigens [128]. Receptors involved in apoptotic cell uptake (at least uninfected apoptotic cells) may divert the dead cells into vesicles that have a paucity of TLRs. Another apparent question that remains to be answered in light of studies by Schulz [122] and Chen [124], is the fate of infected apoptotic cells: do they behave like their uninfected counterparts or do they undergo rapid uptake similar to the one observed after internalization of bacteria?

22.3 Mechanisms of Crossprocessing and Crosspresentation

What are the mechanisms that allow access of exogenous antigens into the presentation pathway that is normally restricted to antigens produced within the cell? Initially, investigation focused on entry of exogenous antigens into the classical pathway; however there is data suggesting that processing of exogenous antigens may be carried out in a self-sufficient phagosome-derived compartment, although this requires further confirmation. Here we list possible cellular mechanisms of crosspresentation that have been described over the last decade. (An overview of cellular mechanisms in crosspresentation is shown in Fig. 22.1).

22.3.1 Entry into the Classical Endocytic Pathway

Classical presentation of endogenous self- or pathogen-derived antigens involves their processing into peptides by cytosolic proteases, mainly proteasomes, followed

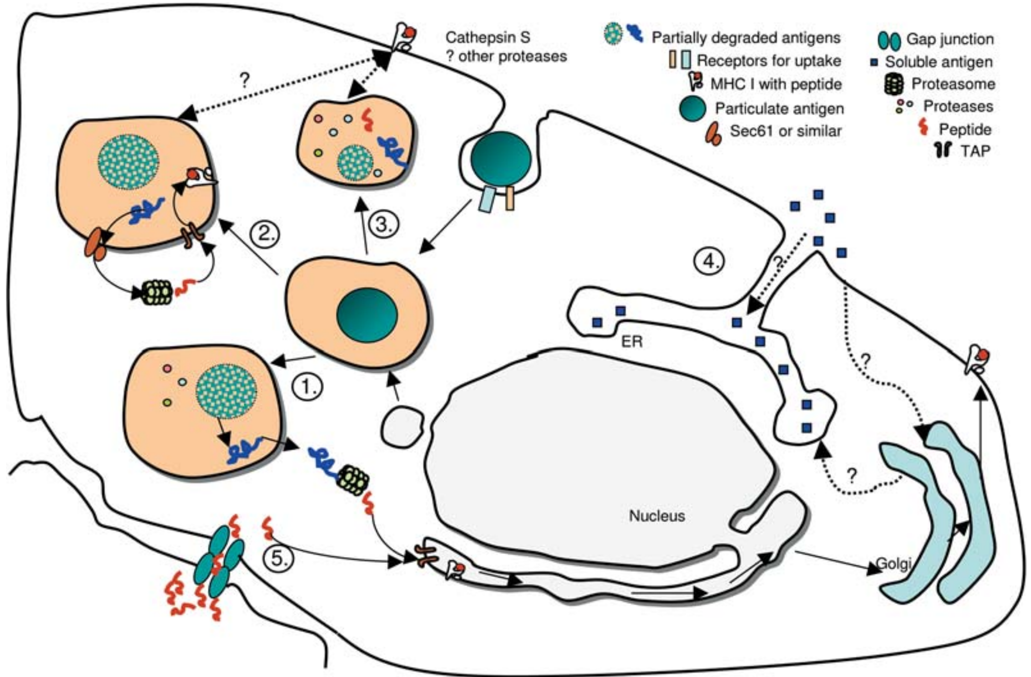

Fig. 22.1 Mechanisms of crosspresentation. Particulate antigens enter the antigen presenting cells most efficiently by receptor mediated endocytosis. Endosomes containing the ingested antigen then acquire selected endoplasmatic reticulum (ER)-derived elements and, after initial processing within the endosome, the antigens exit the late endosome and enter the cytoplasm. They then enter the classical pathway and are processed in a similar manner as endogenous antigens (1). Alternatively, the antigens may remain in the endosomes that acquire selected characteristics of ER and are fully processed in close viscinity of the phago-endosome. They re-enter the phago-endosomes and are there loaded onto MHC class I molecules (2). Whereas in the former cases the antigen processing involves transporter of antigen processing (TAP), this is not always required. Albeit less efficiently, the vacuolar processing pathway (3) may rely on vacuolar proteases, especially cathepsin S, to generate peptides that are loaded onto MHC class I molecules within the vacuole. A special pathway may be in place for soluble antigens (4) which by yet unidentified mechanisms directly access the ER. Gap junctions may permit the transfer of already processed peptides from donor to acceptor cells (5).

by transport via TAP molecules (transporter associated proteins) into the lumen of the endoplasmic reticulum (ER), where peptides bind to newly synthesized MHC class I molecules. MHC molecules bearing peptides are finally transported via the Golgi apparatus to the plasma membrane. Exogenous antigens such as IC, soluble proteins, apoptotic or necrotic cells and antigen-coated beads can also access this pathway from phagolysosomes and endosomes, following transfer into the cytosol [8, 49, 129, 130]. In human DCs, dying cells are captured into endocytic vesicles. After acidification and maturation of the vesicles, the antigens within the ingested apoptotic cells undergo proteolysis by cathepsins and are transported into the cyto-

sol for further processing [130]. Cathepsin D has been identified as one such protease which participates in the crosspresentation of influenza infected apoptotic cells.

Crosspresentation is crucially dependent on actin cytoskeleton rearrangements [130, 131], a process that is extensively controlled by Rho GTPases [132] such as Rac1 or Cdc42. Transgenic mice expressing a dominant negative form of Rac1 under the CD11c promoter have reduced numbers of $CD8\alpha^+$ DCs. The remaining DCs were substantially impaired in the *in vivo* uptake of exogenously delivered OVA loaded $\beta 2m^{-/-}$ splenocytes. A consequence was decreased crosspresentation and crosspriming of OVA-specific OT I cells [131]. In accordance, it was shown previously that Rac 1 is activated upon binding of apoptotic cells to the integrin receptor $\alpha v\beta 5$ [133]. Murine DCs that were transfected with vaccinia encoding constitutively active Rho GTPases increased the uptake of OVA protein and its presentation to $CD4^+$ T cells. The process was abrogated by toxin B from *Clostridium difficile* which inactivates Rho, Rac and Cdc42 [134]. As attention is being turned to the role of the cytoskeleton and its regulatory proteins in crosspresenting mechanisms, we will learn more of its involvement in the differential trafficking of internalized antigens.

22.3.2
Phagosome–endosome Compartment

An alternative pathway of processing exogenous antigens in the context of MHC class I presentation has recently been proposed by several groups. Phagosomes become enriched with components of ER whereby the resulting phagosome–endosome compartment with adjunct proteasomes forms a self-sufficient compartment for loading of MHC class I molecules with peptides ("one stop processing").

The first studies indicating that ER is involved at early phases of phagocytosis of crosspresentation came from the group of Desjardins. Gagnon and co-authors [135] showed that in macrophages, but not neutrophils, the ER donates the membrane to the forming phagosome, which in the process of maturation continues to incorporate elements of ER. This mechanism is involved in the uptake of inert particles, as well as microorganisms such as *Salmonella*, a bacterium that triggers crosspresentation [136]. Not surprisingly, in the next year three publications followed that showed involvement of ER membranes in phagosomes containing material to be crosspresented. The group of Desjardins [137] extended their previous studies by showing that in mouse macrophages, proteasomes are closely associated with phagosomal-ER compartment at its cytoplasmic side. Additionally they showed that the membranes of the compartment are enriched with TAP, another essential component for antigen presentation. When OVA containing latex beads were engulfed into phagosomes, the $CD8^+$ T cell-restricted epitope SIINFEKL could be detected with a specific antibody, with maximal expression 1–2 h after phagocytosis. In an independent study, Guermonprez et al. identified a similar process [138]. The authors used a complex procedure to isolate and purify phagosomes from phagocytosing murine DCs. Selected ER residential proteins such as

Sec61/Sec62, calnexin and TAP were recruited soon after formation of the phagosome. Additionally, components of MHC loading machinery: tapasin, ERp57, calreticulin and heavy chains of MHC class I molecules were detected in the phagosome. Exposure of OVA-latex beads to DCs resulted in functional OVA-MHC class I complexes and stimulation of CD8 T cell-restricted eptiope specific OT I T cells.

A similar mechanism may take place in human DCs. Ackerman et al. [139] showed that either primary human DCs or DC-like cell line KG-1 phagosomes as well as micropinosomes, all contain elements of the MHC loading machinery.

Lizée et al. [140] have identified a conserved sequence in the cytoplasmic domain of MHC class I heavy chain that apparently directs the molecules into the phagolysosomeal compartment. In mouse $CD11c^+$ DCs, deletion of a tyrosine residue in this domain alters routing of MHC class I molecules and consequently prevents crosspresentation of VSV and Sendai virus antigens and activation of specific CTLs.

Taken together, these studies imply that phagosomes enriched by elements donated by the ER could be a self-sufficient compartment for crosspresentation. According to the model, Sec61 would translocate protein segments derived from exogenous antigens into the cytoplasm where they would be processed into peptides by the closely adjacent proteasomes and retrotransported by TAP into the lumen of the phagosome. On the luminal side of phagosomes, the translocated peptides are loaded onto MHC molecules. The last step, the route by which peptide-MHC accesses the cell surface, has not been resolved, but may involve recycling MHC class I molecules.

However, the drawback of these studies is that they have relied on the use of artificial forms of antigen (latex beads), and therefore it remains unclear whether such ER-phagosomes are involved in the actual processing of apoptotic cells. Further studies will be essential to confirm the existence of this pathway and to determine whether it applies to physiological sources of antigens.

22.3.3
A Special Mechanism for Soluble Antigens?

A detour from the self-sufficient phagosome based mechanism of crosspresentation was recently proposed by the Creswell group, who made the observation that soluble antigens can gain access to the perinuclear ER compartment [141]. They used protease-resistant human β2m as a source of exogenous soluble antigen. They first showed that the β2m co-localized with the ER-marker calnexin but not with the lysosomal marker LAMP-1 or Golgi marker GM130. MHC class I molecules are not folded correctly in the absence of β2m, and consequently are eventually degraded in the ER. When exogenous β2m was added to DCs derived from the bone marrow (BM) of β2m-deficient mice, the expression of correctly folded MHC class I molecules on the cell surface was restored. The MHC molecules correctly folded with the help of exogenous β2m were even capable of stimulating OVA specific T cells when DCs were infected with vaccinia virus encoding OVA. Taken together, these data indicate, that the exogenous antigen accessed the ER to attain functionality in the described experimental system.

With this pathway, several questions remain to be addressed, including the mechanisms that deliver the soluble antigens to the ER: whether this involves the transient luminal continuities between pinosomes and ER [135] and/or retrograde transport through the Golgi [142].

22.3.4
Tap Dependence and Endocytic Exchange Mechanism (Vacuolar Pathway)

As discussed, exogenous antigens can be internalized into endocytic vesicles [8, 101, 143], and crosspresented onto MHC I molecules via different pathways. One involves the transfer of antigens into the cytosol from the phagosome, followed by further degradation in the cytosol by proteosomes and then transport via TAP into the ER. In addition, vacuolar processing (possibly in an ER-phagosome structure associated with TAP molecules), may permit "one-stop" processing [137, 138]. Finally, pre-processing in vacuoles may take place before access to the cytosol or the ER through an endocytic exchange mechanism. In most but not all of these cases, crosspresentation onto MHC class I molecules would be significantly TAP-dependent. However, there are now several examples of TAP-independent pathways. Below we review some of these data, how they relate to TAP-dependent pathways and how they might be integrated into the current models of crosspresentation.

Using a bone marrow chimera system, Huang et al., first convincingly demonstrated a role for TAP-dependent crosspriming *in vivo*. CT26 tumor lines (H-2d) expressing the influenza NP protein were delivered to lethally irradiated F1 strains (H-2 bxd), which had been reconstituted with wild type or $TAP^{-/-}$ (H-2b) bone marrow. As expected, crosspriming occurred in the $TAP^{+/+}$ reconstituted F1 mice, which developed a NP-specific, H-2Db restricted CTL response. However, no crosspriming was observed when chimeras were reconstituted with $TAP^{-/-}$ bone marrow. These studies supported a dominant TAP-dependent pathway for *in vivo* crosspriming to tumor cells [144]. Studies from other investigators confirmed that tumor and other cell associated antigens are also crosspresented, at least partly, by the TAP-dependent pathway [49, 71].

Subsequent studies by Sigal and Rock showed that $TAP^{+/+}$ bone marrow derived APCs were also essential for crosspriming to vaccinia and polio virus [145, 146]. The initiation of responses to LCMV and influenza virus also required bone marrow derived DCs, but unlike polio virus, both TAP-dependent and -independent pathways participated. Interestingly, the authors found that the TAP-independent pathway was much less efficient than the TAP-dependent route, on the order of 10–300-fold less [146]. Although the pathway by which the TAP-independent crosspriming takes place was not described, it was speculated that viral epitopes in exogenously acquired viral antigens were processed and presented to MHC class I molecules in endosomes. It is possible that the exogenous antigens are accessed as virus infected apoptotic cells. Consistent with this concept, Albert et al., have described both TAP-dependent and -independent pathways for the crosspresentation of influenza virus infected apoptotic cells by murine DCs. In their model they suggest that processed antigens are needed for efficient crosspresentation by the TAP-independent pathway [147], as crosspresentation was inhibited when apoptotic

cells were pre-exposed to lactacystin (a proteosome inhibitor) before delivery to $TAP^{-/-}$ DCs.

Recent studies by Shen et al., have demonstrated a TAP independence of the vacuolar pathway of cross presentation, depending upon the physical form of the antigens. OVA incorporated into microspheres is apparently crosspresented by both TAP-dependent and -independent pathways. The authors showed that in the presence of $TAP^{-/-}$ DCs, this form of OVA, was inhibited from being cross presented by leupeptin (an inhibitor of cysteine proteases), This was further supported by the finding that cathepsin S, but not cathepsin L or B deficient DCs were deficient in the crosspresentation of microsphere complexed OVA. Furthermore, double $TAP^{-/-}$ and cathepsin $S^{-/-}$ mice completely failed to cross present the microsphere complexed OVA, indicating a critical role for cathepsin S in TAP-independent processing pathways. Cathepsin S also contributes to the crosspriming of cell-associated antigens as $TAP^{-/-}$ cathepsin $S^{-/-}$ animals failed to crosspresent OVA expressed in cells to OVA-specific T cells. Finally, a role for cathepsin S was identified in the crosspresentation of influenza viral epitopes. Compared to the overall response, however, the vacuolar pathway seemed less significant than the TAP-dependent pathways [148]. Larsson et al., have also reported on a partial requirement for cathepsins in the crosspresentation of virus infected apoptotic cells by human DCs. Blocking of aspartic proteases with pepstatin, led to inhibition of crosspresentation. Since proteosome inhibitors also partly blocked presentation, it is conceivable that some of the influenza antigens are first partially processed in the phagosome, before access to the cytosol. However, in this study it was not possible to rule out a TAP-independent, but cathepsin-dependent pathway [130]. These studies point to the possibility that different cathepsins may be involved in generating relevant peptides from various antigens.

TAP-dependent and -independent pathways have also been described using other forms of antigens for example HSP complexes containing antigenic peptides [149, 150]. In addition to being necessary for peptide transport from the cytosol to the ER, or retrograde transport from the cytosol to ER-phagosomes, TAP dependence may rely on the fact that MHC molecules in $TAP^{-/-}$ cells have reduced stability at phagolysosomal pH. Chefalo et al., showed that both tapasin and TAP were necessary for MHC-I to bind ER derived stabilizing peptides so that they could achieve the stability needed for peptide exchange in acidic vacuolar processing compartments [151]. These studies indicate that the endocytic vacuolar processing pathway requires the transfer of appropriately assembled MHC class I molecules from the ER.

22.3.5
Transfer of Peptides via Gap Junctions

A recently described pathway of crosspresentation involves the transfer of peptides from infected to neighboring cells by virtue of gap junctions [109]. Gap junctions are channels composed of connexin molecules in cell membranes that connect the neighboring cells for the purpose of ion, messenger and nutrient exchange. Apart from the limit in maximal size of transferred molecules (MW<1K) the mechanism

seems to be unspecific. Neijssen et al. [109] show that a similar mechanism is used to also transfer peptides directly from the cytoplasm of one cell to the cytoplasm of a neighboring cell. Initially they used a cell line which does not express gap junctions. They then stably transfected the line with connexin 43 (Cx43). By comparing the control and Cx43 transfected lines and with the use of a chemical inhibitor of gap junction transfer, they were able to show that transfer of fluorescently labeled peptides from one to another cell depends exclusively on the use of gap junctions. Subsequently, a similar approach was used to show the transfer of immunogenic peptides and acquisition of antigen presentation ability of the acceptor cells.

The physiological relevance of this pathway remains unclear. As the cytosol is full of peptidases, only a small fraction of peptides would persist in the cytosol long enough to be transferred to the neighboring cells. Further destruction in the acceptor cell would ensure rapid dilution of the peptides, giving gap junction transferred peptides a very limited spatial spread. It has been proposed that peptides arising from infection of cells, could be transferred to adjacent non-infected cells and recognized by T cells. Such selective transfer could form a “buffer zone” that would contribute to self-limitation of infection. It is not clear whether the transfer of peptides via gap junctions plays any role in crosspriming. DCs do express proteins involved in gap junction formation [152], however, given the likely inefficiency of gap junction transfer, a major role in crosspriming seems unlikely.

22.4 Physiological Relevance of Crosspresentation

Let us finish by briefly tackling the subject that is more thoroughly discussed elsewhere in this publication. Why did the complicated mechanism of crosspresentation evolve? What is the physiological relevance of crosspriming? Whereas crosspresentation and crosspriming can take place in response to tumors [115, 153, 154], viruses [76, 77, 155–164] and bacteria [136, 165–167], the relevance in comparison to direct priming has been questioned [168, 169]. However, several circumstances call for the existence of alternative mechanisms of priming, including priming to tumor antigens which themselves are poor stimulators of T cells. Perhaps an even more apparent example is that of microbes that have developed immune escape mechanisms, for example abrogation of presentation on MHC class I molecules on infected cells but where nevertheless efficient long term immunity is still established (e.g. to CMV). These examples support the notion that mechanisms such as crosspresentation are immunologically relevant and sometimes indispensable [139].

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23
A Systems Biologist's View of Dendritic Cell–Cytotoxic T Lymphocyte Interaction

Burkhard Ludewig and Gennady Bocharov

23.1
Introduction

CD8$^+$ cytotoxic T lymphocyte (CTL) responses represent a major immune mechanism of protection against many viral infections and tumors through the killing of altered (infected or malignant) cells [1]. Viruses generally provide signals to the immune system, either on the surface of infected cells or on antigen-presenting cells, that permit CD8$^+$ T cells to recognize virus-encoded peptides through their T-cell receptor (TCR). Viruses that induce an early and efficient CD8$^+$ T-cell response, such as the lymphocytic choriomeningitis virus (LCMV) in mice [2, 3] or the human immunodeficiency virus (HIV) [4, 5] tend to infect professional antigen-presenting cells such as DCs and macrophages. Importantly, DCs can be infected by viruses independently of a specific receptor [6], a finding that supports the notion that the general susceptibility of DCs to infection with a wide range of viruses may represent an important prerequisite for efficient CTL priming against viruses with more restricted tissue tropism [7].

Virus localization and transport, governing the kinetics of appearance of the viral antigens in different compartments of the body, determine whether CTL reactivity is induced, maintained, or aborted. Primary virus infection normally leads to CTL dynamics that have three distinctive phases: clonal expansion, contraction, and persistence of memory cells. Both the magnitude and the duration of the CTL response are tightly regulated at the single cell and the CTL population level. The early steps of T-cell proliferation/differentiation are still poorly characterized. For example, it still remains to be resolved how fast the effector function is acquired, or whether the activated CTLs follow "sequential" or "parallel" differentiation routes to become effector or memory CTL. Nonetheless, a successful/protective CTL response depends critically on the availability of specific antigens, efficient delivery of these antigens and their optimal presentation to the T cells within secondary lymphoid organs.

DCs are known for their remarkable potential to induce specific T-cell responses [8]. The extraordinary efficacy of DC to prime immune responses is shown by

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the fact that only 10^2 to 10^3 antigen-presenting DCs in the spleen are sufficient to elicit protective levels of antiviral CTL activation in mice [9]. Furthermore, a series of experimental studies in mice demonstrated that tumor-specific CTL can be induced by adoptive transfer of antigen-presenting DCs [10, 11]. However, the success of DC-based immunotherapeutical treatment of human cancer is still limited [12], most probably because the complexity of the regulation of the DC–CTL interaction is far beyond simple dose-effect type regulation schemes and its sensitivity to various factors is not yet completely understood. For example, DCs positively regulate immune responses by mediating antigen influx and antigen presentation in secondary lymphoid organs. However, they are rapidly lost during the cognate interaction with fully activated effector T cells [13].

The ability of DCs to induce protective CTL responses to a broad spectrum of pathogens (viruses and intracellular bacteria) under a wide range of conditions, implies that the interaction of DCs and CTLs is a *robust* biological system. This interaction is regulated at the single-cell level by a complex signal transduction and gene activation machinery and at the cell-population level by various modes of communication (e.g. cytokines, cognate interaction between MHC and TCR molecules, and recognition of particular surface molecules by cognate receptors) in different spatial compartments. The interaction of DC and CTL therefore is based on a highly regulated set of spatiotemporal interactions with the outcome depending on a large number of intra- and inter-cellular physical and biochemical parameters. Because of the inherent complexity of these processes, we follow here a *"systems biology"* analysis of the structure, dynamics and the operating principles that permit DCs to induce protective CTL responses, with a particular focus on the robustness of this phenomenon. We will (i) consider the components and structures that are central in the DC–CTL interaction; (ii) discuss the mechanisms that underlie specific DC–CTL interaction outcomes; and (iii) describe the sensitivity of the major patterns of CTL responses to variations in the interacting components or environment conditions. In our view, this conceptual framework might help to provide a consistent theoretical basis for transforming "disordered networks" of factors that potentially influence the interaction of DCs and CTLs into an "ordered hierarchy" of relevant parameters ranked according to their quantitative effects on the production of reliable and robust CTL responses.

23.2
Deciphering the Systems Biologist's Approach

"Systems biology" represents a framework for analysis and interpretation of the structure and dynamics of complex biological phenomena. Although it has a long history [14, 15], it is because of the recent advances in technologies for high-throughput and quantitative measurement of the interactions at the level of genes, proteins, cells, tissues, organs and systems that the interest in system-level approaches to biological phenomena has been renewed [16]. In theoretical immunology, the need for a systems approach was appreciated in late 1970s [17]. In experi-

mental immunology, however, it has not become a universally accepted principle in the interpretation of immune phenomena yet, despite the fact that this approach has productively been used to address the issues of (i) the protection unit – the module of humoral immunity selected by evolution [18]; (ii) immune system recognition of antigen [19]; (iii) regulation of the immune responses [20]; and (iv) principles involved in effective operation of the immune system [21].

Systems biology focuses on the analysis of the structure, dynamics, design principles and control methods of biological systems in order to understand how robustness is achieved. Robustness means the ability to maintain stable functioning despite various perturbations [22]. It is recognized as an essential feature of biological systems and a conserved organizing principle in biology. Indeed, in a variety of biological systems ranging from genetic switches to physiological reactions, the robustness of their state and function to external and internal perturbations has been established [23]. The systems theory suggests that robustness is achieved through modularity, feedback, redundancy and structural stability. It is important that acquiring robustness against a certain set of perturbations is associated with fragility to other perturbations, which are outside the conventional set. This aspect of complex systems performance is considered to be related to the conservation of the fragility principle, and this issue has been discussed in detail in recent reviews by Kitano [23] and Csete and Doyle [24].

The ability of DCs to induce protective CTL responses to a broad spectrum of pathogens under a wide range of conditions with minimal immunopathological damage is critical in assuring the survival of the host. This implies that this physiological reaction of the immune system, namely the interaction of DCs with CTLs, represents a robust biological system. Therefore, "a systems biology" analysis of the various parameters of DC-mediated induction of CTL responses represents a coherent approach to deal with the complexity of the issue. This requires, however, a change in the type of questions that are being addressed before reviewing the current knowledge about DC–CTL interaction. The following section therefore introduces the key elements of the systems biology analysis of immune responses which govern the intrinsic robustness of DC–CTL interaction.

23.2.1
Modularity and Protocols

Modules are the components, parts or subsystems that contain the following features: (i) they have identifiable interfaces to other modules; (ii) they can be modified somewhat independently; (iii) they maintain some identity when isolated or rearranged; yet (iv) they derive additional identity from the rest of the system [24]. The modular approach greatly facilitates the conceptual understanding of the structure of complex systems. The meaning of modules varies depending on the level (from proteins and genes to pathways and networks and finally to tissues and organs) and the type (physical or logical/functional structure) of analysis. For example, the organs in the immune system, or the leukocyte subsets (DCs, T- or B-cells) represent two straightforward examples of physical modules. On the other

hand, the antigen-processing pathways for peptide loading of MHC class I or class II molecules can be viewed as examples of two functional modules.

The modular structure of complex systems is thought to enhance their robustness by preventing spread or amplification of local perturbations [25]. In the case of the DC–CTL interaction, modules can be specified at various levels. For the purpose of our analysis we distinguish three basic modules: the thymus as "educational" module, the secondary lymphoid organs as "activation" module and the peripheral organs as "surveillance" module. Fig. 23.1 shows the modularity feature of the DC-dependent CTL maturation from a progenitor cell into a functional effector CTL.

Modular organization implies the need to understand the rules that prescribe interfaces or protocols between modules, permitting system functions that could not be achieved by isolated modules [24]. From a structural perspective, every cell in our body presents on the cell surface almost every cellular protein in complexes with MHC class I molecules providing information to the immune system about the cellular interior. This *MHC-restricted presentation* gives an example of a protocol, which is used in the DC–CTL interaction at the single cell level. For the TCR-mediated signaling event, there are additional molecules that must be integrated into consideration, such as CD28-CD80/CD86 or CLTA-4-CD80/CD80 interactions which represent second order parameters in the induction of CTL responses.

At the immune system level, it is the DC that provides the interface between the surveillance and activation modules (see Fig. 23.1). Indeed, the antigen, which is outside organized lymphoid tissues, is ignored unless it gets trapped by the antigen-presenting cells and brought into the secondary lymphoid organs [26]. There are some other general rules for the induction of immune responses, e.g. that the immune system responds to a *rapid perturbation* in its homeostasis – a term that includes an increase in antigenic stimulation and the requirement for specialized tissue milieus [20].

From the functional perspective, the ability to generate a biphasic signal response is an essential design constraint on the modular implementation of complex systems in biology [27]. The well-known low- and high-zone tolerance [28] or recently shown bell-shaped relationship between the peak CTL expansion and virus growth rate in LCMV system [29], confirm that the immune system is adjusted to respond to a certain optimal level in the activation signal, whereas the response to signals outside the optimal range is not induced.

23.2.2
Feedback Control

The concept of feedback control is central to the functioning of complex systems since it provides the robustness to external disturbances and variation of internal components [24]. The positive feedback acts to amplify small disturbances creating switches and breaking homogeneities, whereas the negative feedback serves to maintain patterns/states. It is noteworthy that in multilevel hierarchical systems, the task of the higher-level regulators is not to control, but rather to produce a coherent behavior/functioning of the lower level modules [30].

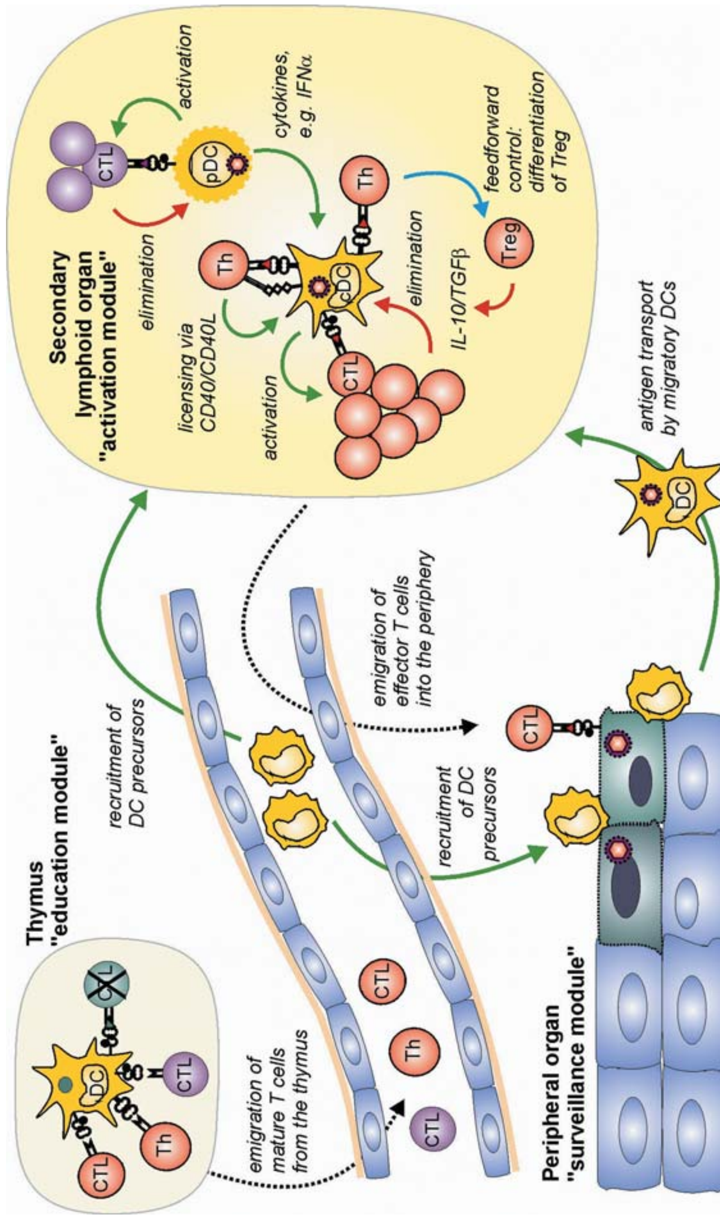

Fig. 23.1 Systems biologist's view of DC–CTL interactions. *Modularity* in the complex system of (direct and indirect) DC–CTL interactions is represented by different organizational modules: thymus ("educational module"), secondary lymphoid organs ("activation module"), and peripheral nonlymphoid organs ("surveillance module"). Different DC subsets, e.g. conventional DCs (cDC) versus plasmacytoid DCs (pDC) provide heterogenous *redundancy* and ensure homogenous outcome, that is, activation of CTL and secretion of stimulating cytokines. Positive *feedback* mechanisms (e.g. chemokine-driven recruitment of DC precursors into peripheral organs or secondary lymphoid organs, or CD40-mediated licensing of DCs) are indicated by green arrows. Negative feedback control loops such as elimination of DCs by effector CTL are depicted in red. Generation of regulatory Th cells (Treg) can be seen as feedforward control (blue arrow).

Immune processes have to be tightly regulated which is achieved by multiple interconnected control loops, implementing various types of feedback and feedforward controls. At the cellular level, the combination of negative (disturbances attenuating) and positive (disturbances amplifying) feedback loops is considered to be fundamental for generating the TCR-dependent signaling apparatus, which is both highly sensitive and discriminatory [21]. Individual cells tune and update

their activation thresholds through changes in the co-stimulatory molecules expression, or intracellular kinetic competition between activating and deactivating signals [20].

The positive feedback is essential in all growth processes. in the immune system it serves to break up initial homogeneity of the lymphocyte repertoire and to adapt the repertoire to the pattern of antigenic perturbations experienced during the life of the host. In the context of the DC-mediated CTL priming the DC functions to amplify the antigen-specific CTL clone(s). It has been suggested [21] that mutually amplifying positive feedback interactions between CTL and DCs lead to a maximal response once an initial signal rises beyond a low minimal level (see Fig. 23.1 and Section 23.3.1). Conversely, there is growing evidence which suggests that CTL-mediated elimination of the antigen-presenting DC is probably a key process in the downregulation of adaptive immune responses so that the immune reaction does not become an immune overreaction [13, 31, 32]. The amplification effect of antigen-presenting DC on the CTL combined with the negative feedback from effector CTL on DC numbers implies that the cell population dynamics of the CTL–DC system *in vivo* reflects a predator–prey type of interaction [33]. This combination of positive and negative controls regulating the DC mediated CTL priming is depicted schematically in Fig. 23.1.

23.2.3 Redundancy

Redundancy means that multiple components/modules with equivalent or overlapping functions are present in the system and can substitute each other [23]. It allows the complex system to respond to a knockout by using alternative components ensuring robustness to component failure. The homogeneous redundancy, based on multiple copies of identical components, is considered to be effective when stimuli are targeted to a specific component. In general, the heterogeneous redundancy mediated by functionally close components is thought to ensure better protection against common mode failures. A combination of homogeneous outcome based upon heterogeneous implementation is an essential design feature of robust systems.

The immune system provides a rich source of examples of both homogeneous and heterogeneous type of redundancy at the systemic, cell population and molecular level; e.g. (i) its spatial structure represents an interconnected network of primary and secondary lymphoid organs with many similar yet distinct features maintaining a diverse repertoire of lymphocytes, (ii) $CD4^+$ and $CD8^+$ T cells display some similar functions, e.g. both can produce the effector cytokine interferon-gamma (IFNγ) or eliminate circulating target cells in a cognate interaction [34, 35], (iii) the chemokine network is a highly redundant system [36].

In the context of the DC-mediated induction of protective CTL responses, the redundancy is apparent in several ways. Two examples are presented in Fig. 23.1: (1) Redundancy on the CTL side is achieved in the form of multiple CTL clones which recognize the same epitope and are heterogeneous in the recognition features

(binding affinity, on/off rates). The polyclonality and multispecificity of CTL responses are thought to be important in the control of viral infections, such as HBV [37] or HIV [38]. (2) Redundancy at the level of DC reveals itself in the existence of at least two distinct DC populations, known as conventional and plasmacytoid DCs, and a range of subsets with remarkable functional plasticity facilitating avid reactions against the pathogen.

23.2.4 Structural Stability

The system is called structurally stable if it preserves patterns of dynamics in spite of some perturbations in its parameters [39], this implies the existence of in-built resistance to particular perturbations. Concepts of stability are concerned with the effect on the system's behavior of perturbations in the system, e.g. kinetic parameters, cell numbers, environmental factors. The analyses of how systems behavior changes when perturbations are introduced into the system and which built-in mechanisms promote the stability are the focus of experimental immunology. Specifically, it has the aim to unravel the parameter variations, module knockouts, mutations, etc., which change the specific behavior. For example, the overall interaction of high-avidity CTLs with DCs displays an intrinsic structural stability once a certain threshold number of DCs presenting a particular peptide had entered a secondary lymphoid organ [33]. A further trait of the inherent structural stability in the interaction between DCs and CTLs is illustrated by the robust induction of antiviral CTL responses even in the absence of organized T-cell zones in secondary lymphoid organs. Mice lacking the chemokine receptor CCR7 [40] or its ligand chemokine CCL19 and CCL21 [41] display significant defects in the correct intralymphoid organs positioning of DCs and CTLs. Despite the lack of functional T-cell zones and a shift of DC–CTL interactions to the marginal zone, both CCR7-deficient [42] and *plt/plt* mice [43] exhibit only minor impairments in the induction of protective antiviral immune responses. These studies provide evidence for the relative insensitivity of DC–CTL interactions to particular perturbations.

23.3 From Systems Biology to DC–CTL Immunobiology

The frequency of specific CTL precursors (CTLp) for a distinct peptide/MHC complex is rather low, with approximately 1 in 100 000 in mice [44, 45] or humans [46]. In response to infection with LCMV, for example, these CTLp expand by a factor of 10^4–10^5, i.e. 100–300 CTL precursors (CTLp) per spleen expand to up to 10^7 effector CTLs specific for a single epitope [45, 47]. The doubling time during this proliferation period is around 6–8 h [44, 48]. Proliferation of CTLp is accompanied by differentiation into effector cells which possess the ability to secrete cytokine such as IFNγ [47], upregulate the expression of cytolytic granules and effector molecules such as perforin [49], and gain the ability to emigrate from secondary lymphoid

organs into nonlymphoid organs through downregulation of CCR7 and CD62L [42]. The maximal expansion of CTL is followed by a contraction phase with death of >90% of the specific CTL effectors and the establishment of a rather stable memory population [50, 51].

It is important to note that the induction of CTL responses by vaccination with DCs expressing a high-affinity LCMV peptide follows similar kinetics as seen in the original viral infection. The maximal CTL expansion following priming with DCs is reached between days 6 and 8 [9, 33], the doubling time of the responding CTL population is 6–8 h, and the overall expansion scale is in the range of 10^4–10^5-fold [33]. CTLs elicited by DC-priming are able to perform *ex vivo* [9] and *in vivo* [11] cytolytic activity and protect mice from systemic and peripheral viral challenge [9, 52]. CTL frequencies remain elevated in the primed host, indicating establishment of a stable memory population [52]. These studies suggest that priming of antiviral CTLs is almost exclusively mediated by DCs presenting the immunodominant viral peptides. Indeed, recent studies support this view [53–55]. This section introduces the essential components involved in the interaction of DCs and CTLs, and discusses – in the light of a systems biologist's approach – the most important concepts describing DC-induced CTL responses.

23.3.1
Dynamics of CTL Activation and Differentiation

Antigenic stimulation of CTLs results in well-ordered changes in the transcriptional program of the cell defining naïve, effector and memory populations [56]. However, the differentiation pathways along which CTL effector and memory populations develop still remain controversial. Essentially, two different schemes, progressive versus programmed differentiation, have been put forward to explain the dynamics of CTL differentiation. Both views share the distinction between naïve (N, antigen unexperienced), effector (E, fully differentiated), effector memory (T_{EM}, CCR7- and CD62L-negative), and central memory (T_{CM}, CCR7- and CD62L-positive) T cells [51, 57]. The concept of programmed differentiation suggests that T cells differentiate along the following linear sequence: $N \rightarrow E \rightarrow T_{EM} \rightarrow T_{CM}$ [51]. Evidence for this view comes from studies showing that the brief antigen exposure (only 24 h) was sufficient to program activation, expansion and differentiation of naïve CTLs into effector CTLs [58, 59]. Importantly, the differentiation program proceeded in an antigen-independent manner towards the memory state [59]. Further analyses suggested that memory CTL (de)differentiate in an hierarchical manner from T_{EM} into T_{CM} [60, 61].

A contrasting model suggests that depending on the signal strength accumulated in the single T cell, hierarchical thresholds of proliferation and differentiation have to be passed [57]. Signal strength is defined here as the cumulative number of MHC–TCR interactions over time, and the overall co-stimulation received via surface molecules and soluble factors such as cytokines. On the basis of these considerations, a nonlinear, progressive differentiation of naïve T cells into either T_{CM},

T_{EM} or E following the interaction with DCs has been suggested [62]. Although this concept has been derived mainly on the basis of *in vitro* data [63–66], it is most helpful in explaining the large functional and phenotypical heterogeneity of both $CD4^+$ and $CD8^+$ T cells that is observed during viral infections [67, 68]. Progressive differentiation of T cells is most probably the basis for the observation that self-renewal of Th cells is inversely correlated with the differentiation status [69]. Likewise, CTL of the T_{CM}–type generated after LCMV infection have a greater capacity to proliferate after re-encounter with the antigen compared with T_{EM} CTL [60]. These findings and the concept of progressive differentiation are in accordance with the view that the balance between proliferation and differentiation depends on the strength and the quality of the antigenic impact [70, 71].

It is apparent that the partly conflicting models mentioned above might require re-evaluation, validation and/or refinement in order to come even closer to a realistic view of DC-induced CTL activation and differentiation. Intravital microscopy offers – to some extent – the possibility to "watch", for example, the interaction of CTLs with DCs in a higher resolution on a single cell level in the native anatomical context [54]. Results from a recent study indicate that there is an initial phase of the DC–CTL interaction where CTLs first scan multiple DCs which results in a reduction of their motility and an initial activation sequence with upregulation of distinct surface markers. Following the initial phase, rather stable DC–CTL clusters are formed for about 12 h, leading to the differentiation into cytokine-secreting effector CTLs [72]. CTLs then detach from the DCs and start to interact again shortly with antigen-bearing DCs, followed by CTL proliferation [72]. It is most likely that a rather undifferentiated subpopulation of DC-activated CTLs commences with a further proliferation–differentiation cycle whereas more differentiated CTLs emigrate into peripheral tissues and/or exert their effector function within secondary lymphoid organs. This view is supported by adoptive transfer studies showing that IFNγ producing Th cells failed to give rise to proliferating progenitors whereas non-IFNγ-producers exhibited self-renewal capacity [73].

23.3.2
Multiple Levels of Positive and Negative Feedback Control

Feedback control represents an important organizing principle of complex systems. The proper balance between positive and negative feedback loops is of utmost importance for the efficient response to perturbations and the maintenance of system stability. Regulation of antigen influx to secondary lymphoid organs via DC represents an important positive regulatory loop for the generation of CTL responses, whereas the elimination of DCs by effector CTLs can be seen as integrated negative feedback. Activation of DCs during the early interaction with CTLs or Th cells ("licensing") is part of a positive amplification mode. Negative feedforward control of excessive CTL responses, on the other hand, is most likely achieved through the concomitant induction of IL-10 producing regulatory Th cells.

23.3.2.1 Managing DC Recruitment and Antigen Translocation

DCs display two distinct functional phenotypes whereby immature DCs in non-lymphoid organs are specialized for uptake and processing of foreign antigens and DCs within secondary lymphoid organs represent more the mature phenotype with the ability to stimulate naïve T-cell responses. Immature DCs are localized in rather high densities in epithelial sites of the body surface, such as skin epidermis and the mucosae of the respiratory, gastrointestinal and urogenital tracts. Importantly, immature DCs are rare in "immunologically privileged" sites such as the central nervous system or the testis unless they are recruited to these sites by inflammatory stimuli. Immature DC display a distinct repertoire of inflammatory chemokine receptors (CCR1, CCR5, CCR6) [74] which bind a broad set of inflammatory chemokines, including RANTES, MCP-3, MIP-1α, MIP-1β, MIP-5. The ability of DC precursors and immature DCs to respond to inflammatory chemokines is probably relevant for their accumulation in peripheral tissues during the early phase of an inflammation. The localization of DCs within secondary lymphoid organs is controlled by the constitutive chemokines CCL19/ELC, CCL21/SLC, and CXCL13/BLC which are differentially expressed in the B and T-cell zones [75]. The chemokines CCL19 and CCL21 act via the CCR7 receptor and are capable of attracting naïve T cells and mature DCs [40, 76]. Importantly, pathogen-induced inflammation may accelerate both the recruitment of DC precursors into peripheral tissues [77, 78], and migration of DCs into secondary lymphoid organs [79, 80]. Thus, antigen sampling in the periphery and influx into secondary lymphoid organs is positively regulated through the distinct migration pattern of DCs: first, by increased recruitment of immature DCs to peripheral inflamed sites, and second, by promoting DC translocation to secondary lymphoid tissues.

23.3.2.2 Elimination of DCs by Effector CTL

The fact that DCs do not recirculate after they have homed to secondary lymphoid organs [81, 82] suggests that their life cycle is locally regulated during T/DC interactions. Whereas T helper cell-derived signals such as CD40/CD40L interaction activate DCs and/or maintain their viability [83, 84], other T cell-derived cytokines, such as IL-10 [85] or TGFβ [86] may inhibit DC function or induce apoptosis of mature DCs [83]. Antigen-presenting DCs may be rapidly lost during the cognate interaction with fully activated Th cells [87] or during massive nonspecific immune stimulation such as LPS treatment [88]. Furthermore, the cognate interaction with effector CTLs leads to the elimination of antigen-presenting DCs [31, 32]. The fact that loss of APCs due to killing by effector CTLs is a hallmark of infection with noncytopathic viruses such as LCMV [3, 89] suggests that elimination of APCs *in vivo* serves as a negative feedback mechanism to prevent exaggerated immune activation.

23.3.2.3 Rapid Amplification of Signals through Molecular "Ping–Pong" Interactions

Following encounter with an infectious agent, CTL responses are generated swift-

ly. Importantly, the induction of initial CTL responses against pathogens – with only few exceptions – is not dependent on the presence of Th cells [90, 91]. Such highly efficient activation is most likely set in motion through positive feedback loops with staggered back-and-forth signaling between DCs and the responding CTLs. Essentially, these processes start with cognate TCR–MHC interactions. Signaling via costimulatory molecules potentiates the APC function of DCs by upregulation of CD80 and CD86, which reciprocally enhance the activation of the T cells through triggering of CD28 [92, 93]. It is important to note that the function of these "second order" co-stimulatory signals depend on the "first order" signal delivered by the antigen.

The finding that CTL induction in case of limiting antigen availability [94–96] depends on the concomitant activation of Th cells indicates that the overall signal strength during the initial DC–CTL interaction (MHC–TCR contact plus co-stimulatory signals) has to reach a certain threshold. For example, amplification of the signal strength delivered to the CTLs by antigen-presenting DCs can be achieved through contact-dependent, CD40-mediated activation of the DCs by $CD4^+$ T cells, a process that has been termed "conditioning" [95] or "licensing" [97]. Since $CD8^+$ T cells can efficiently induce maturation of DCs, even in the absence of CD40 [98], it is conceivable that three-cell-type clusters of DCs, CTLs, and Th cells are necessary to generate efficient T-cell responses in cases of low antigenic impact [99].

23.3.2.4 **Limiting the CTL "Overshoot" through Feedforward Control**

The immune system reacts, as outlined above, to strong perturbations, caused by rapidly increasing amounts of antigen and concomitant inflammation. The initial delay in the control of the pathogen has to be seen as a result of an initially undeveloped specific immune response. However, the transiently overshooting pathogen load is, eventually, cleared from the tissues by an overshooting immune response [20, 71]. In order to minimize overshoot-associated immunopathological damage, the immune system employs potent feedforward control mechanisms both on the molecular and the cellular level.

Co-signaling molecules are important for the control and modulation of T-cell responses to antigen [93]. Whereas the co-stimulatory molecule CD28 is constitutively expressed on both $CD4^+$ and $CD8^+$ T cells, the co-inhibitory molecule CTLA-4 is only expressed on T cells following their activation. The engagement of the B7-family members CD80 and CD86 on the responding APCs exerts negative feedback on the proliferating T cells, e.g. by preventing IL-2 production and arresting cell cycle progression [100, 101]. "Programming" of the negative CLTA-4 signal during the CD28-dependent initial activation of T cells represents a typical feedforward control mechanism. Importantly, other members of the CD28 family such as PD-1 exert inhibitory functions during the activation of T cells [93]. Since the ligands of CD28, CTLA-4, and PD-1, i.e. CD80/CD86 and B7-H1, are expressed on DCs, it is reasonable to assume that it is the temporal sequence of the expression of co-stimulatory and co-inhibitory signals that regulates the magnitude and the differentiation pattern of T cells during their interaction with DCs.

Feedforward control on the cellular level is most likely a task of regulatory Th cells (Treg) which exert suppressive effects mainly via the co-inhibitory surface molecule CTLA-4 and the cytokine IL-10 [102]. Just like CTLA-4 expression, the production of IL-10 appears to be programmed early during the activation of Th cells [103]. Release of IL-10 is a hallmark of chronically activated Th1 and Th2 cells and thus, is important to avoid immunopathological damage in the host [104]. Taken together, the three-cell-type cluster interaction between DCs, CTLs and Th cells generates not only positive amplification loops during the initial T-cell proliferation via "licensing" of the DCs, but also implements feedforward control through CTLA-4 expressing and IL-10 secreting regulatory Th cells which contribute to counterbalance the CTL overshoot.

23.3.3
DC Subsets Provide Redundant Activating Signals

Robustness of complex systems does not only require sophisticated feedback mechanisms but also redundant modules and protocols to avoid disastrous system failure. We suggest that the existence of various DCs subtypes with distinct but largely overlapping functions secures redundancy for the swift activation of immune responses against pathogens and is essential for the induction of T-cell responses against tumors.

The exact differentiation pathway of DC precursors are still, to some extent, controversially discussed, however, it is now widely accepted that DCs arise from both multipotent myeloid and lymphoid bone-marrow precursors [105]. Furthermore, monocytes can give rise to immunostimulatory DCs *in vitro* under the influence of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 [106–109] or *in vivo* following their migration into secondary lymphoid organs [110, 111]. Langerhans cells (LCs), the prototype of immature DC, reside mainly within stratified squamous epithelia of the skin and mucous membranes were they closely attach to neighboring epithelial cells via an E-cadherin- and Ca^{2+}-dependent mechanism [112]. Separate precursors of LCs can be distinguished in bone-marrow cultures by their phenotype, by involvement of a recognizable monocyte stage and by their requirement for TGF-β [113]. Plasmacytoid DCs (pDCs) or interferon-producing cells can be differentiated from $CD4^{+}CD11c^{-}CD3^{-}$ precursors present in human blood or tonsillar lymphocyte preparations into DC-like cells using CD40 crosslinking [114]. The murine counterpart of the human pDC has been characterized recently by Trinchieri and colleagues [115]. Murine pDCs or interferon-alpha-producing cells (IPC) [115] and human pDCs [116, 117] secrete large amounts of type I interferon after viral stimulation and therefore most likely restrict initial viral replication and participate in the generation of antiviral Th1 responses [118]. The diverse differentiation pathways of DCs are indicative for a highly redundant system securing the generation of an array of immunostimulatory cells.

The existence of DCs that may co-develop with T cells from a distinct lymphoid progenitor cell subset in the thymus indicates that thymic CD8α$^+$ DC may develop along a separate intrathymic differentiation pathway. Furthermore, both thymic and splenic DCs can differentiate from thymic "low CD4 precursors" [119], whereas bone marrow precursors produce both CD8α$^+$ and CD8α$^-$ DC populations in mouse spleen [120]. Initial *in vitro* experiments showed that CD8α$^+$ or CD8α$^-$ DC subsets did not differentiate into one or the other [121] which led to the hypothesis that CD8α$^+$ and CD8α$^-$ DC subsets represent separate DC lineages. However, recent studies indicate that CD8α$^-$ LC can acquire CD8α upon maturation by CD40 ligation [122] and that CD8α$^-$ DCs differentiate into CD8α$^+$ DCs *in vivo* [123, 124] suggesting that CD8α expression is associated with the differentiation of DCs. Furthermore, the finding that monocytes can differentiate into both CD8α$^-$ and CD8α$^+$ DCs *in vivo* [110] makes it rather unlikely that CD8α is an exclusive marker for a distinct DC lineage.

The initial two-lineage-concept based on the phenotypical differences in CD8α expression led to the hypothesis that the two DC subsets may possess opposing functions, i.e. that "myeloid" CD8α$^-$ DCs induce T cells responses, whereas (nowadays so-called) "lymphoid" CD8α$^+$ DC in the periphery induce deletion of potentially self-reactive T cells [125], e.g. via Fas/FasL-mediated apoptosis during DC–T cell interactions [126]. This view is supported by findings of Ferguson et al. [127] and Belz et al. [128] that CD8α$^+$ DCs mediate the induction of cross-tolerance. However, studies showing that both CD8α$^-$ and CD8α$^+$ DC induce vigorous T-cell responses after adoptive transfer into naive mice [129, 130], that CD8α$^+$ DC are responsible for priming of immune responses against exogenous antigen [131], and that CD8α$^+$ DC are the major cells involved in priming of antiviral CTL responses [132–134] suggest that induction of immune reactivity is the default pathway after DCs have encountered sufficient amounts of antigen.

The DC-lineage concept had been complemented by studies suggesting that plasmacytoid DC precursors favor exclusively the development of Th2-type responses whereas "myeloid" DCs derived from monocytic precursors determine Th1-type immune responses [135]. However, subsequent studies have shown that pDCs respond swiftly with secretion of large amounts of type I interferon following encounter with a broad range of viruses [136] which fosters the generation of antiviral Th1 cell responses [118]. Furthermore, peptide-pulsed pDC possess the ability to directly elicit effector CTL secreting IFNγ [137] indicating that, following appropriate stimulation, pDCs are capable of stimulating proliferation and differentiation of protective CTL responses.

The concept that distinct DC subsets or committed DC lineages may be endowed with distinct functions [138], e.g. induction versus tolerance or Th1- versus Th2-induction, is further challenged by recent studies that provide evidence for the high plasticity among DC subsets [139, 140]. It appears that the overall effect of antigen dose together with the quality of the inflammatory stimulus, e.g. stimulation via different Toll-like receptors (TLRs), determines whether pDCs and conventional DCs drive either a Th1 or and Th2 response [139]. Furthermore, LCMV infection

elicits an apparent conversion of $CD11c^{+}B220^{+}CD11b^{-}$ pDC precursors into $CD11c^{+}B220^{-}CD11b^{+}$ "conventional" DCs with the ability to efficiently present antigen to T cells and produce IL-12 in response to TLR-4 ligation [140]. Taken together, these studies support the view that the different DC subsets with their interchangeable functions and their high plasticity represent an adaptable sentinel cell system that promotes effective and swift responses against invading pathogens.

23.3.4 Tuning of Dendritic Cell Activation

Activation of T cells depends on the kinetics of antigen appearance in secondary lymphoid organs, the "first order" parameter, and "second order" parameters such as stimulation via co-stimulatory surface molecules or inflammatory cytokines. Hence, an acute systemic perturbation with a rapid assembly of DCs delivering large amounts of antigen and T cells in the T-cell zone of lymph nodes or the spleen results in immune activation. Importantly, the immune system does not discriminate whether the antigen is "self" or "foreign" as long as sufficient amounts of antigen are presented for a minimal period of time by mature DCs [141, 142]. In case of low level perturbation, i.e. constant presentation of small amounts of antigen, T cells may be activated transiently, but without the development of a burst-like response [143]. This transient activation without extensive proliferation and differentiation into effector T cells results, however, in the adjustment of the activation thresholds of the individual cell [70]. An elevated activation threshold through repeated low-level antigenic stimulation will essentially result in the "ignorance" of the antigenic stimulus, as long as the stimulus remains at subthreshold levels. This model of "activation threshold tuning" implies that the excitation levels of T cells constantly adapt to the overall stimulation supplied by the antigen-presenting cells, mainly DCs, in secondary lymphoid organs [20, 70, 143]. Whereas tuning of T-cell activation threshold results in a constantly changing excitability, it appears that the initial activation threshold for DCs remains constantly at low levels, i.e. despite variations in the mode of activation due to context-dependency, it is an intrinsic feature of DC physiology to respond rapidly and profoundly to subtle perturbations in their environment.

23.3.4.1 Excitement through Pattern Recognition

Viruses and other pathogens display particular molecular signatures ("pathogen-associated molecular pattern") that are recognized by specific "pattern recognition receptors" [144]. DCs express a vast array of such receptors, including TLRs and C-type lectins. Whereas C-type lectins facilitate mainly binding of pathogens to and their ingestion by DCs [145], it is the triggering of intra- and extracellular TLRs that leads to rapid activation and maturation of DCs [146]. Maturation of DCs under the influence of TLR stimulation includes upregulation of co-stimulatory molecules, increased loading of MHC class II molecules, improved crosspresentation of exog-

enous antigens, and increased production of immune-stimulatory cytokines such as type I interferons and IL-12 [147]. Consequently, TLR-mediated signaling to pDCs and conventional DCs greatly enhances their ability to prime CTL responses [134, 137, 148], partially via concomitant licensing of Th cells [97]. Importantly, both human and mouse DC subsets express nearly the complete array of all known TLRs [146]. Thus, stimulation of DCs through TLRs and other pattern recognition receptors generates rapid pathogen-specific innate immune responses and conditions DCs for efficient stimulation of adaptive T-cell responses.

Two distinct features of TLR-mediated stimulation of DCs are particularly noteworthy. Namely that the activation of DC following contact with a TLR ligand *in vivo* occurs within only a few hours, and that this activation eventually diminishes the lifespan of the DCs [88, 149], the rapid apoptosis induced by TLR ligation can be – to some extent – counterbalanced during the early cognate interaction with T cells [149]. Thus, DCs represent a tunable cellular switch that integrates a large array of positive and negative signals both from the innate and the adaptive immune system.

23.3.4.2 **DC Tuning and Tolerance to Self-antigens**

It is apparent that the DC system with its different, functionally redundant subsets that all respond avidly to pathogen-associated innate stimuli, is highly efficient in activating T-cell responses. Infection-associated immunopathological damage is minimized by the above-mentioned negative feedback and feedforward control mechanisms. An important question, however, remains to be discussed, namely whether and how DCs contribute to establishment of self-tolerance. The most prominent view – at the moment – is that constitutive expression of self-antigens by immature DCs is associated with tolerance to self-antigens [150]. Indeed, tissue-derived self-antigens can be found in DCs [151, 152]. Furthermore, targeting of antigens to DCs in the absence of pro-inflammatory stimuli results in tolerance [153, 154]. However, presentation of significant amounts of self-antigen by DCs in secondary lymphoid organs – even in the absence of inflammatory signals – neither results in deletion of autoreactive T cells nor does it lead to autoimmunity [155]. Furthermore, *in vitro* and *in vivo* approaches show that even mature DCs can tolerize T cells [156, 157]. To resolve this discrepancy, a further refinement of the DC maturation scheme has been suggested with DCs being either semi-mature [157] or mature, but not licensed, to drive full differentiation of effector T cells [158]. Following this line of reasoning, the decision whether self-reactivity is induced or not depends on rather minuscule changes in the phenotype of a particular DC subset.

The demand for robustness in the interaction of DCs with T cells, i.e. efficient activation of pathogen-specific responses with minimal "collateral" damage, makes it difficult to envisage that, for example, partial upregulation of CD80 or CD86 or slight increases in IL-12 production by DCs would be the decisive switch that determines immune reactivity against self-antigens. We prefer to follow a more global approach with the "spatiotemporal" view of the regulation and homeo-

stasis of immune responses [20, 21, 26]. Accordingly, peripheral self-antigens that are expressed below a certain threshold remain immunologically ignored because they do not reach secondary lymphoid organs [26]. Tissue antigens that reach secondary lymphoid organs in a steady state may – to some extent – induce T-cell activation with few autoreactive effector cells which, however, will not elicit autoimmune disease. Steady state influx of antigen to secondary lymphoid organs and presentation by DCs should eventually lead to activation threshold tuning in the responding T-cell population that keeps autoimmunity in check [20]. These rather simple rules explain why Th-cell responses against even abundantly expressed self-antigens such as collagen, cardiac myosin, or myelin basic protein can be easily induced once the antigen is delivered at supra-threshold levels with appropriate inflammatory stimuli, e.g. via subcutaneous application of a few hundred micrograms of purified antigen in complete Freund's adjuvant. Furthermore, in DC-based immunotherapy of tumors with DCs being loaded with MHC class I peptides, efficient priming of CTL responses against rather abundant self determinants (i.e. tumor antigens) is achieved if appropriate amounts (supra-threshold levels) of antigen are delivered in the right kinetics.

23.4 Conclusions

The induction of protective CTL responses by DCs represents a robust biological system as it ensures protection of individuals against the vast majority of pathogens and helps to maintain immune surveillance of tumors. Novel vaccination approaches for the improvement of CTL responses against infectious agents and tumors should be based upon the systems biology analysis of the robustness of DC–CTL interaction as well as the fragility of the pathological steady states. The framework presented should help to provide an economic conceptualization of *in vitro* and *in vivo* observations to avoid unnecessary complexities (e.g. divergent DC subsets or intermediate maturation stages). Thus, structure, dynamics, design and control principles of DC-induced CTL responses represent important issues that require the attention of further research efforts.

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IX
Dendritic Cells Cross-talk with Other Cell Types

24
Dendritic Cells and Natural Killer Cells

Magali Terme and Laurence Zitvogel

In the last 30 years, dendritic cells (DC) have been studied with regard to antigen-specific immune response and adaptive immunity. Recently, a key role of the DC has been highlighted: their capacity to control innate immunity by triggering NK-cell activation and the role for this interaction in T-cell priming. In this chapter, we will address different questions: (1) which DC subset interacts with NK cells? (2) in which location? (3) how is DC/NK interaction counter-regulated? (4) what is the role of DC-mediated NK-cell activation for $CD4^+$ T and CTL priming? (5) What is the potential pathophysiological relevance of the DC/NK cross-talk? Since this book focuses on DC biology, a short introduction on NK cells is necessary to approach our topic.

24.1
Introduction on NK Cells

NK cells represent the major population of the effectors of the innate arm of immunity. NK cells are large granular lymphocytes characterized by the absence of CD3 and the expression of FcγRIII/CD16 and CD56 in humans and NK1.1 in mice [1]. These cells are key players in immunosurveillance and in the defense against viral infections and tumors. In humans, NK cells are found in lymph nodes and at lower frequency in blood [2]. Lymph nodes harbor 40% of total lymphocytes and NK cells represent 5% of total lymph node lymphocytes, while blood contains 2% of total lymphocytes and NK cells represent 5–15% of total blood lymphocytes. Therefore, lymph node NK cells are, in the absence of infection and inflammation, ten times more abundant than blood NK cells [2]. In mouse, NK cells are mainly found in spleen, but are also detected in lymph nodes and blood and in nonlymphoid organs such as liver, lung, placenta, bone marrow.

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NK cells have been firstly described as "non-MHC-restricted" cells. However, MHC Class I molecules present on the surface of tumor cells play a critical role on the inhibition of the NK cells. Besides, an inverse correlation has been established between the expression of MHC class I molecules on target cells and the susceptibility to NK-cell lysis [3, 4]. This correlation has led to "the missing self hypothesis" which has been confirmed by the characterization of MHC Class I specific inhibitory receptors. But some MHC Class I^+ tumor cell lines are killed by NK cells because of NK-cell activating receptors, such as NKG2D, which allow NK cells to overcome inhibitory signals generated by MHC Class I molecules on tumor cells. NK-cell receptors have recently been reviewed [5–9]. Thus, NK-cell function is tightly regulated by the integration of activating and inhibitory signals initiated by the engagement of cell surface receptors.

Activated NK cells are able to lyse infected or tumor cells, or secrete cytokines. They can directly kill target cells or cells covered with antibodies by the antibody-dependent cellular cytotoxicity (ADCC). Perforin/granzyme exocytosis [10] and death receptor engagement [11–13] are the two major mechanisms used by NK cells to induce target cell apoptosis. Activated NK cells can also secrete immunoregulatory cytokines, such as IFNγ, TNFα, IL-13, GM-CSF [14]. Cytotoxicity and cytokine secretion have been shown to be dependent on different NK-cell subsets. Indeed, in humans, two different NK-cell subsets have been identified by cell surface density of CD56. The majority of blood NK cells (90%) are $CD56^{dim}$ and express high levels of CD16, whereas $CD56^{bright}$ $CD16^{dim}$ NK cells are found at minor levels [15]. $CD56^{dim}$ NK cells are more cytotoxic than $CD56^{bright}$ NK cells [16], whereas $CD56^{bright}$ NK cells are involved in cytokine and chemokine production [14].

Recently, a provocative article demonstrates the capacity of mature human NK cells expressing high levels of MHC class II and co-stimulatory molecules after IL-2 activation to uptake and present particulate antigens or immune complexes and to stimulate antigen specific T cells *in vitro* [17]. However, the relevance of this observation for *in vivo* priming of naïve T cells in the setting of a viral infection remains to be shown.

24.2
Activation of NK Cells by DC

The original description of the existence of the DC/NK-cell interaction has been provided by our laboratory. Fernandez et al. [18] showed that injection of Flt3-L, a DC growth factor, or adoptive transfer of DC induced eradication of AK7 mesothelioma tumors. This antitumor effect was dependent on NK cells. Furthermore, *in vitro* bone-marrow-derived dendritic cells or the D1 DC cell line stimulated with TNFα could stimulate NK-cell effector functions (cytotoxicity and IFNγ production). Thereafter, several groups were interested in the study of the interaction between DC and NK cells. The regulation of NK-cell activation by DC is being slowly unraveled in the mouse and human settings, for both cytolytic and secretory activities as a function of the stimuli received by DC.

24.2.1 NK-cell Activation and DC Subsets

Gerosa et al. [19] have shown that stimulation of peripheral blood mononuclear cells with some Toll-like receptor (TLR) ligands, such as Poly I:C (for TLR3) or R848 (for TLR7–8), results in IFNγ secretion. NK cells represent the majority of IFNγ producing cells, in association with NKT cells and γδT cells. But these cells do not produce IFNγ directly upon stimulation by TLR ligands, except for TLR2 and TLR3 ligands that have been described to directly activate NK-cell functions [20–22]. The presence of $HLA\text{-}DR^+$ antigen presenting cells, and particularly dendritic cells (DC), is required to stimulate NK-cell IFNγ production [19]. However, not all DC subsets are able to stimulate NK cells. Myeloid DC stimulated by TLR ligands are able to stimulate both IFNγ production and cytotoxicity. Several *in vitro* human studies have highlighted the capacity of mature myeloid DC (monocyte-derived DC (MD-DC) and $CD34^+$-derived DC) stimulated by LPS, a TLR4 ligand, to activate resting NK cells what results in the enhancement of cytotoxicity and IFNγ secretion functions, proliferation and upregulation of CD25 and CD69 on NK cells [23–28]. On the other hand, plasmacytoid DC (pDC) pretreated with viruses or TLR9 ligands trigger only NK-cell cytolytic function [19]. By contrast, Langerhans cells fail to induce NK-cell stimulation [29].

In mouse, as in humans, myeloid DC trigger both NK-cell effector functions. Indeed, bone-marrow-derived DC (BM-DC) or splenocytes-derived D1-cell line stimulated by LPS or TNFα are able to activate NK-cell IFNγ production and cytolytic function [18, 30–32]. In contrast with human pDC, plasmacytoid DC isolated from MCMV-infected mice can not only activate cytotoxicity but also IFNγ production by NK cells [33].

24.2.2 Molecular Mechanisms of the DC-mediated NK-cell Activation

Several studies have highlighted the fact that the maturation state of DC influence their capacity to activate NK cells. Immature DC require a maturation stimuli, such as LPS, TNFα, *Mycobacterium tuberculosis* or IFNα, to activate resting NK cells [23–26]. However, we will describe later in this chapter that in some circumstances pathophysiological stimuli can promote DC-mediated NK-cell activation without inducing complete DC maturation.

Both soluble factors and cell–cell contacts are involved in NK-cell triggering by DC [23–26, 28]. Mature DC are a major source of IL-12 [34] and other cytokines, such as IL-18, that are mainly implicated in the stimulation of NK-cell IFNγ production [19, 23, 35]. Myeloid DC or pDC stimulated respectively by TLR3 ligands and by viruses or TLR9 ligands secrete type I IFN that is mostly involved in activation of NK-cell cytotoxicity [19]. Cell-to-cell contacts and synapse formation between DC and NK cells are also needed [23, 35]. Formation of stimulatory synapses between DC and NK cells depends on lipid raft mobilization and remodeling of cytoskeleton and promotes the polarized secretion of preassembled stores of IL-12

by DCs towards the NK-cell [35]. The synaptic delivery of IL-12 by DC was found to be required for IFNγ secretion by NK cells, as assessed using inhibitors of cytoskeleton rearrangements and transwell experiments. Besides, Langerhans cells fail to induce NK-cell activation presumably because they do not produce IL-12 and express IL-15Rα [29].

While cytokines play an important role in mature DC-mediated NK-cell activation, cell surface receptors are also involved. Then, Jinushi et al. [36] have shown that IFNα treatment of MD-DC induce the upregulation of MHC-Class I related chain-A and B (MICA/B), two ligands for the NK-cell activating receptor NKG2D, that contribute to NK-cell triggering. IL-15-derived DC seems to be implicated in the upregulation of MICA/B on MD-DC after IFNα exposure [37].

In murine studies, most DC are derived from bone marrow and treated with TLR4 ligands such as LPS or *E. coli* bacteria. Following TLR4 triggering, BM-DC propagated in the absence of IL-4 transiently produce IL-2 [38, 39] and stimulate NK-cell cytotoxicity and IFNγ secretion by two different mechanisms. IL-2-derived DC is involved in the activation of IFNγ production while type I IFN produced by DC triggers NK-cell cytotoxicity as in humans [32]. By contrast, IL-12 and IL-18 do not have a key role in this setting. *In vivo*, inoculation of *E. coli* promotes IL-2 secretion by DC that contribute to NK-cell activation [32]. However, when IL-4-propagated BM-DC are exposed to LPS, they do not produce IL-2, because of the inhibitory role of IL-4 on IL-2 production [40]. By contrast, upon LPS treatment BM-DC generated in the presence of IL-4 upregulate IL-15Rα and produce IL-15 that contribute to the activation of NK-cell cytotoxicity [31]. In this setting, IL-12 acts in synergy with IL-15 and IL-15Rα molecules to induce NK-cell IFNγ secretion [31]. Using IL-12-deficient mice, Borg et al. [35] have confirmed that IL-12 have a pivotal role in the induction of IFNγ production by mature BM-DC.

In the absence of TLR4 stimulation, IL-4-propagated BM-DC are also able to activate NK-cell functions, unlike BM-DC generated in the absence of IL-4 [30]. The triggering receptor expressed on myeloid cells-2 (TREM2) associated with KARAP/DAP12 adaptor molecule is upregulated on BM-DC by IL-4 and involved in DC-mediated NK-cell activation [30].

24.3
Reciprocal Interaction of DC and NK Cells

Communication between DC and NK cells is not unidirectional but bi-directional and should really be considered as a dialog. Thus, not only do DC have the ability to activate NK cells, but activated NK cells are also able to stimulate maturation or lysis of DC. According to Piccioli et al. [25], the outcome of the NK-DC interactions is tightly regulated by the NK/DC cell ratio. Indeed, at a low NK/DC ratio DC maturation prevails, while at a high NK/DC ratio DC lysis occurs [25].

24.3.1
DC Maturation Induced by NK Cells

Once NK cells are activated, they can interact with immature MD-DC and induce, or at least augment, their maturation characterized by the upregulation of CCR7, MHC Class II molecules and co-stimulatory molecules, such as CD80, CD86, CD83, but also by enhanced antigen-presenting capacity, and production of IL-12 [23, 25]. This NK cell-dependent DC maturation requires both cell–cell contact and TNFα secretion [23, 25]. In the presence of suboptimal stimuli, e.g. CpG, activated NK cells favor pDC maturation leading to type I IFN and TNF production [19]. In a mouse model, we have highlighted that NK cells, that have been activated by IL-4-propagated BM-DC, can in turn promote maturation of BM-DC characterized by the upregulation of CD80 and CD86 molecules on DC surface. This maturation is in part due to the TREM2/KARAP/DAP12 pathway, at least for the upregulation of CD86 [30]. The relevance of this phenomenon *in vivo* has been demonstrated in a mouse model of MHC ClI^{lo} tumor. Indeed, ClI^{lo} tumors induce NK-cell activation that in cascade stimulate DC activation, characterized by IL-12 production [41].

Thus, activated NK cells can induce DC maturation either directly or in synergy with suboptimal levels of microbial signals. NK cell-induced DC activation is dependent on both TNFα/IFNγ secretion and a cell–cell contact. As shown by Vitale et al. in a human setting [42], the NK cell-mediated DC maturation involving the release of TNFα by NK cells depends on the triggering of the NK-cell receptor NKp30 and is counter-regulated by KIR and NKG2A inhibitory receptors. In a mouse tumor model, the group of Mocikat [43] has been able to show a critical role for IL-12 produced by endogenous DC following IFNγ secretion by activated NK cells, underscoring a link between innate immunity and cross priming of CTL [43] (as discussed below).

Therefore, NK help might be critical for optimal DC activation and subsequent induction of T-cell responses in conditions where inflammation is poor but where NK-cell activation could occur through direct recognition of target cells. In particular, this may be relevant to defense against cancer, as discussed below.

24.3.2
Lysis of DC by Activated NK Cells

The relevance of the NK cell-mediated DC lysis is more intriguing. Immature DC are preferentially killed by NK cells [24, 25, 44, 45], but after maturation by microbial stimulation or cytokines, DC become resistant to NK-cell killing [44–46]. This is due to the upregulation of MHC Class I molecules on DC upon maturation that protect DC from NK-cell lysis [46, 47]. Killing of immature DC is confined to a small NK-cell subset expressing the CD94/NKG2A inhibitory receptor [48]. Thus, DC that express high levels of HLA-E, the CD94/NKG2A ligand, are protected from NK-cell lysis. However, a small subset of NK cells expressing low levels of NKG2A is also able to lyse mature DC [48]. Activating receptors, such as NKp30 and maybe others, are also needed to kill immature DC [24]. A recent *in vivo* study

performed in mice has shown that immature DC are eliminated by NK cells via a pathway dependent on the TNF-related apoptosis-inducing ligand (TRAIL) [49]. Depletion of NK cells or neutralization of TRAIL function during immunization with immature DC loaded with tumor antigens significantly enhanced cognate T-cell responses, suggesting that the lysis of DC by NK cells might limit vaccination efficacy [49].

24.4
Where do DC Meet NK Cells?

24.4.1
In Lymph Nodes

The presence of NK cells in lymph nodes (LN) has been controversial for a long time. But recently, some studies have highlighted the existence of a large amount of NK cells in inflamed and uninflamed lymph nodes [50, 51]. NK cells seem to be ten times more abundant in LN than in blood [51]. In inflamed LN, NK cells have been localized in the T-cell area, using immunohistochemistry and PCR [50]. In uninflamed LN, the presence of NK cells in the T-cell area has been confirmed, including clusters in the parafollicular regions of the T-cell zone [52]. In the T-cell area, DEC-205^{+} DC have also been identified, suggesting that LN might be an essential site of DC/NK-cell encounter [52]. These LN-NK cells are mainly CD56^{bright} and are non cytolytic [51]. The CD56^{bright} NK-cell subset has been described as being the most reactive NK-cell subset to DC *in vitro* [52, 53].

However, a question remains: does DC interact with resident NK cells or with NK cells recruited from blood? Recent studies have shown that LPS-activated MD-DC can interact with resting CD56^{bright} NK cells isolated from LN within 6 h and induce NK-cell IFNγ production [52]. This study suggests that DC could interact with resident LN-NK cells and modulate cytokine secretion thereby regulating Th differentiation. Ferlazzo et al. have suggested that CD56^{bright} NK cells could be a precursor for CD56^{dim} NK cells, because when CD56^{bright} NK cells are cultured with IL-2 during one week they acquire a phenotype similar to CD56^{dim} NK cells [51]. Thus, DC could induce NK-cell to become cytolytic effectors in LN. Another hypothesis is that NK cells could be recruited by DC from blood through high endothelial venules (HEV). In blood, CD56^{bright} NK cells express high levels of CCR7 and CD62L, two molecules involved in the recruitment towards LN. However, we cannot exclude that CD56^{dim} NK cells, which express a lot of chemokine receptors such as the fractalkine receptor CX3CR1, or CXCR3 the receptor for IP-10 or I-TAC, could be recruited from blood through HEV by chemokines secreted by DC.

Different groups have tried to study the recruitment of NK cells by DC in LN in murine models. Our laboratory has been the first to show that when IL-4-propagated or LPS-matured BM-DC are inoculated into the footpad of Nude mice or immunocompetent C57Bl/6 mice, NK cells are activated and their number is enhanced in the draining LN [30]. Adoptive transfer of labeled NK cells showed that NK cells

are recruited from blood through HEV upon mature DC or *Leishmania major* promastigotes injection in footpad [54, 55]. In the same way, IL-4-propagated DC seem to provoke NK-cell recruitment from blood rather than resident NK-cell proliferation, since resident NK cells do not incorporate BrdU (Terme et al., in preparation). Using intravital microscopy, NK-cell behavior has been characterized in LN in the steady state and upon *L. major* infection. At the steady state, NK cells are found in the LN outer paracortex in close vicinity with DC near HEV. But these cells are slow motile, unlike T cells which move rapidly. After *L. major* infection, NK cells rapidly accumulate in the outer paracortex where they can interact with DC and T lymphocytes and secrete IFNγ, but they do not acquire higher motility [55]. Footpad injection of LPS-matured DC trigger NK-cell recruitment in LN in a CXCR3-dependent manner, rather than in a CCR7-dependent manner [54]. These NK cells produce IFNγ that is necessary for Th1 polarization [54]. We will discuss in another chapter the role of DC/NK-cell cross-talk on T-cell polarization and priming.

24.4.2
In the Periphery

DC/NK-cell interaction can also happen at sites of inflammation. The $CD56^{dim}$ $CD16^{+}$ NK-cell subset, that represents the prevalent NK-cell subset in the peripheral blood expresses a chemokine receptor repertoire that allows them to migrate in inflamed tissues. They can migrate in tissues in response to different chemokines, e.g. CCL3, CXCL8, CX3CL1 or CCL22, that are produced by DC or other cells, such as macrophages, endothelial cells or neutrophiles [15, 56]. Thus, NK cells can penetrate into the tissues, where they can encounter resident DC. Moreover, different arguments suggest that DC/NK cross-talk exists in periphery. NK cells have been detected in close contact with DC in Malassezia-induced atopic dermatitis lesion [57], and in imatinib mesylate-induced lichenoid dermatitis [58]. An aberrant $PEN5^{+}$ NK-cell infiltration has also been found in tissues infiltrated with malignant Langerhans histocytosis, suggesting that a dysregulation of the LC/NK cross-talk could participate in chronic inflammation (Borg C., unpublished observations).

24.5
DC/NK Cross-talk and T Lymphocytes

24.5.1
Bridging Innate and Adaptive Immunity

As previously discussed (Section 24.3.1), activated NK cells can induce DC maturation. Thus, NK-cell activation could be crucial for T-cell responses. Mailliard et al. [59] have shown that in the presence of tumor targets and cytokines (IFNα), human NK cells become activated. Those activated NK cells promote the maturation of DC that acquire the capacity to produce IL-12, thereby promoting Th1 polariza-

tion and a $CD8^+$ T-cell-specific response [59] *in vitro*. In a mouse model where the growth of MHC Class I^{lo} tumor targets induced NK-cell activation, IL-12 produced by DC after NK-cell dependent maturation induced a $CD8^+$ T-cell memory response [41].

Other studies have highlighted the fact that IFNγ produced by activated NK cells can influence T-cell responses. Indeed, resistance to *L. major* involves a Th1 response that is dependent on IFNγ produced by NK cells [60]. In the RMA-S-Rae1β tumor model, tumor rejection is mediated by NK cells via a NKG2D-dependent mechanism, but NK-cell activation is also responsible for a secondary T-cell-specific response [61].

A recent and elegant study has revealed that recruitment of NK cells in lymph nodes upon stimulation by LPS-matured OVA-pulsed DC or adjuvants such as Ri-

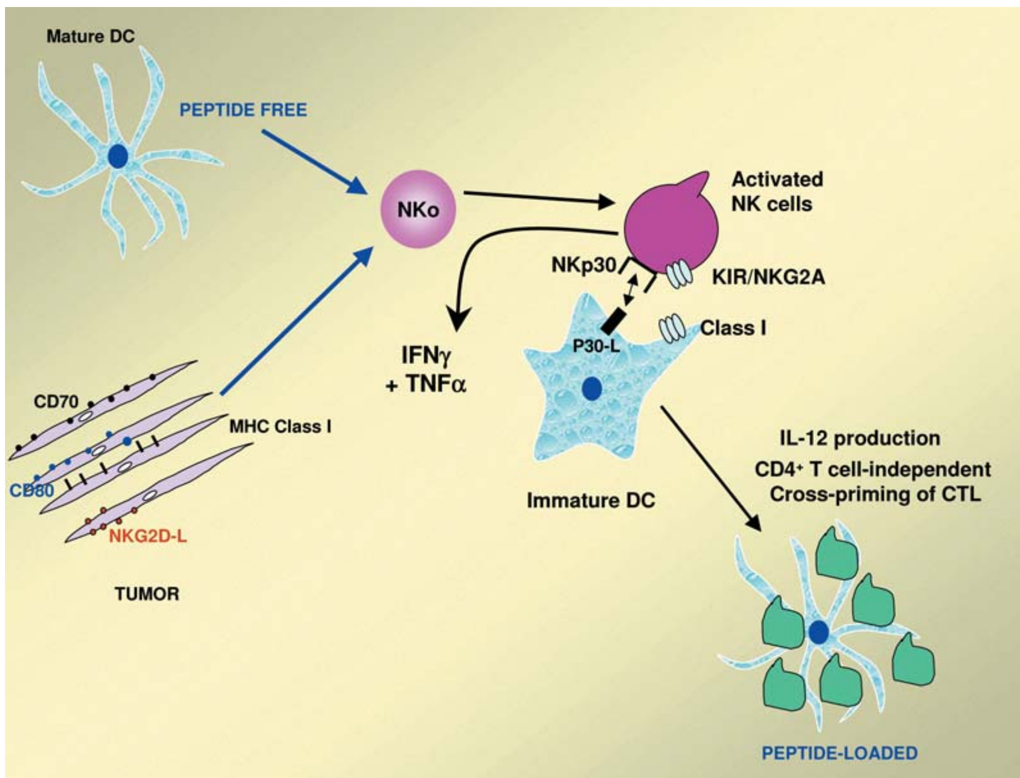

Fig. 24.1 A DC/NK/DC cross-talk leading to efficient cross-presentation of tumor antigens. Tumor cells over-expressing NKG2D ligands or CD70 or CD80 might directly trigger NK-cell activation. Alternatively, exogenous LPS-activated mature DC can turn on NK cells *in vivo*. DC-activated NK cells produce IFNγ which in turn, promotes endogenous DC activation and production of IL-12 and elicitation of CTL cross priming in the absence of $CD4^+$ T-cell help [43]. In the human setting, DC-activated NK cells or IL-2 activated NK cells will induce DC maturation through engagement of NKp30, in a TNF-α dependent-manner.

bi or R848 correlates with Th1 priming [54]. Depletion experiments have shown that NK cells provide IFNγ that is pivotal for Th1 response [54] (Fig. 24.1). However, the role of IFNγ on Th1 response is not really understood and it remains unclear at which level IFNγ can act: on naïve T cells or on host DC.

A recent study [43] assigns a new role for the DC/NK-cell cross-talk in immune responses. The authors demonstrate efficient CTL priming and long term $CD8^+$ T-cell memory against A20 tumor antigens in the absence of $CD4^+$ T-cell help using $CD40^{-/-}$ mature DC, unpulsed with tumor antigens but capable of NK-cell triggering. Their data clearly show that expression of NKG2D ligands on A20 are required for NK-cell triggering, and that IFNγ was necessary for (1) NK-cell activation, (2) activation of endogenous DC and (3) IL-12 production leading to CTL induction (Fig. 24.1).

24.5.2 Modulation of the DC/NK-cell Cross-talk by CD4+CD25+ Regulatory T Cells and Conventional T Cells

Recent advances in our understanding of the biology of regulatory T cells prompted us to determine which impact regulatory T cells (Treg) might have on NK cells and also on DC/NK-cell cross-talk. We have highlighted an inverse correlation between NK-cell activation and Treg in cancer-bearing patients (GIST and melanoma). In humans, Treg directly inhibit activation of NK-cell cytotoxicity and IL-12-mediated IFNγ production, and reduce NKG2D expression on NK cells. This NK-cell inhibition is due to membrane-bound TGFβ [80]. In the mouse model, only activated regulatory T cells (activation by anti-CD3 and anti-CD28) express membrane-bound TGFβ and have the ability to directly inhibit NK-cell cytotoxicity and induce a downregulation of NKG2D on NK cells upon adoptive transfer in Nude mice. Moreover, Treg control NK-cell homeostatic proliferation *in vivo* since Treg cells deficiency or depletion by cyclophosphamide (CTX) or anti-CD25 antibody results in an enhanced proliferation of NK cells [80]. In CTX-treated mice, adoptive transfer of IL-4-generated BM-DC induce an augmentation of NK-cell proliferation in the draining lymph node compared with the contralateral lymph node (Terme et al., in preparation). Moreover, in CTX-treated mice, NK-cell activation in draining lymph nodes upon DC injection is enhanced as assessed by CD69 expression on NK cells and IFNγ production. Thus, Treg inhibit DC-mediated NK-cell activation *in vivo*. *In vitro*, resting Treg inhibit DC-mediated induction of IFNγ production but also NK-cell-mediated DC maturation (Terme et al., in preparation).

Therefore, activated Treg directly inhibit NK-cell cytotoxicity in mouse and human in a TGFβ-dependent manner, whereas resting Treg restrict DC-mediated NK-cell activation. However, some subsets of T lymphocytes are also able to enhance NK-cell activation. In experiments where IL-4 propagated BM-DC were injected in Nude Mice, the number of $DX5^+$ or $NK1.1^+$ NK cells expressing CD69 was increased by 2-fold by 24 h, whereas in immunocompetent C57Bl/6 mice, the number of $CD69^+$ NK cells was augmented by 3–4-fold [30] suggesting a potential role of IL-2 produced by $CD4^+$ T cells in this NK-cell activation [50].

24.6
The DC/NK-cell Cross-talk in Physiopathology

24.6.1
In Infectious Diseases

24.6.1.1 Viral Infections

NK cells contribute to innate defense during certain viral infections, such as infections caused by *Herpes* viruses, hepatitis viruses or HIV [62, 63]. For example, NK cells have a key role in controling murine cytomegalovirus (MCMV) infection [64]. NK-cell cytotoxicity and cytokine or chemokine production are both involved in the defense against this virus, cytotoxicity is predominant in the spleen, whereas IFNγ production occurs in the liver [65]. Not all NK cells seem to be important in the elimination of MCMV. Indeed, the Ly49H activating NK-cell receptor which recognizes the MCMV m157 glycoprotein is restricted to a particular NK-cell subset [64, 66]. CpG motifs, which are ligands for TLR9, are abundant in the genomes of α and β *Herpes* viruses such as HSV and MCMV. DC and pDC have been shown to recognize MCMV through TLR9 [67]. Although DC lose their capacity to stimulate T cells upon MCMV infection, probably because of the loss of their IL-2 production capacity [68], Andrews et al. have highlighted the importance of the DC (especially $CD8\alpha^+$ DC subset) in the expansion of the $Ly49H^+$ NK-cell subset via IL-12 and IL-18 [69]. $Ly49H^+$ NK cells in turn favor the survival of $CD8\alpha^+$ DC [69], confirming the hypothesis that the interaction between DC and NK cells is bi-directional. pDC are also pivotal players in viral infections. During MCMV infection, pDC recognize MCMV through TLR9 and secrete high levels of type I IFN and IL-12 which induce NK-cell activation and promote viral clearance by $Ly49H^+$ NK cells [33, 67]. Upon pDC depletion, type I IFN secretion is dramatically reduced, but other cell types compensate and secrete large amounts of IL-12 guaranteeing high levels of IFNγ and normal NK-cell responses to MCMV [67]. Thus, MCMV recognition via TLR9 promotes pDC, DC and also macrophages to secrete cytokines allowing NK-cell activation and MCMV clearance [67].

NK-cell functions are impaired early in HIV infection [70]. Poggi et al. have shown that HIV-1 Tat, an HIV product, inhibits DC-mediated NK-cell activation by blocking calcium influx and calcium-calmodulin kinase II activation elicited via LFA-1 [71]. Since HIV-1 Tat also inhibits IL-12 secretion by DC, it might be account for the inhibition of NK-cell activation in HIV [72, 73].

NK cells have also been involved in the control of Influenza and Sendai virus [74]. Since plasmacytoid DC secrete type I IFN in response to these viruses, a role for the DC/NK-cell interaction in the control of these infections can not be excluded [75, 76].

24.6.1.2 Bacterial Infections

NK cells play a role in the control of viral infections but also in the clearance of bacterial infection. As we previously discussed, myeloid DC are able to secrete IL-2 during the first hours of bacterial infection [39]. In a murine model of *E. coli*, DC-derived IL-2 promote NK-cell activation and especially IFNγ production which has an important antibacterial function [32]. The number of bacteria in the spleen of mice injected with IL-2-deficient BM-DC is significantly higher than in spleens of mice treated with WT DC, thus IL-2-deficient DC lead to a limited bacterial clearance compared with WT BM-DC [32].

24.6.2 In Cancer

NK cells have been involved in the control of different tumors, such as neuroblastoma, chronic myeloid leukemia (CML) and gastro-intestinal stromal tumors (GIST). GIST cells display typical features of NK-cell sensitivity: TAP-1 deficiency, over-expression of MIC and ULBP (the ligands for NKG2D receptor), loss of MHC class I molecules [58]. Moreover, they are lysed by NK cells from normal volunteers as efficiently as the NK-cell susceptible K562 targets [58].

Our pioneering studies demonstrated the relevance of the DC-mediated NK-cell activation in tumor rejection in a mesothelioma tumor model [18]. Gleevec/STI571, a specific inhibitor of BCR/ABL, c-kit and PDGF-R tyrosine kinases, is used successfully for the treatment of CML and GIST. We have shown that administration of STI571 promote NK-cell dependent antitumor effects in tumor transplantation models using tumor cells that are resistant to STI571 *in vitro* [58]. Antitumor effects are more pronounced when mice are treated with STI571 associated with the DC-growth factor Flt3-L. *In vitro* studies in which GM-CSF and IL-4-propagated BM-DC are incubated with increasing dosage of STI571 highlighted that nanomolar concentrations of STI571 are sufficient to endow DC with ability to stimulate NK cells *in vitro* but do not promote DC maturation. STI571-stimulated DC promote NK-cell IFNγ production independently of IL-12 but require cell–cell contacts. Since identical results are obtained using STI571-treated BM-DC and c-kit-deficient BM-DC, STI571 probably acts by inhibiting the c-kit pathway in DC. Importantly, in GIST-bearing patients Gleevec induce NK-cell activation in 49% of the cases. Gleevec-mediated NK-cell activation is correlated with the clinical outcome. None of the patients who display enhanced NK-cell functions exhibit progressive disease, while all ten patients with refractory GIST have poor NK-cell activity. Moreover, the time to progression is significantly longer in patients with NK-cell activation compared with those who did not show NK-cell activation after Gleevec treatment [58].

Ruggeri et al. [77, 78] have highlighted the role of allogeneic NK cells in graft-versus-leukemia effects (GvL) in acute myeloid leukemia (AML) patients after haploidentical bone-marrow transplantation and in engrafment. But the mechanisms involved in NK-cell activation remain obscure. HLA-C/KIR mismatches between recipient leukemic cells and donor NK cells might not fully account for NK-cell ac-

tivation *in vivo*. Host DC might play a key role for NK-cell activation. Different hypothesis could be envisaged. Indeed, the cytokine storm induced by the myeloablative conditioning could promote DC maturation and NK-cell activation. But with mature DC the risk of graft-versus-host disease (GvHD) development is very high [79]. In chronic myeloid patients, the success of donor lymphocyte infusion in controlling the residual disease remains also poorly understood. Since DC from CML patients bear the BCR/ABL translocation, we hypothesized that BCR/ABL translocation confers to myeloid DC NK-cell stimulatory capacities. Immature monocyte-derived DC from CML patients selectively stimulate NK-cell effector functions unlike immature MD-DC derived from healthy volunteers [81]. Using a mouse CML model obtained by infection of mouse bone marrow by retroviruses bearing the BCR/ABL translocation, we further demonstrated that the BCR/ABL translocation is responsible for the selective NK-cell stimulatory capacities of the CML DC [81]. Surprisingly, although NK cells derived from CML bearing mice display impaired functions and do not respond to IL-2 stimulation, they produce IFNγ in response to BCR/ABL-DC. Since other genetic defects leading to myeloproliferative disorders (JunB- or ICSBP-deficient mice) did not promote NK-cell activation, the BCR/ABL translocation appears unique to confer NK-cell stimulatory capacities. BCR/ABL induce expression of ligands for the NKG2D activating NK-cell receptor on murine and human myeloid DC. STI571, which is a specific inhibitor of BCR/ABL, block the expression of NKG2D ligands on DC. CML-DC-induced killing activity is significantly hampered by anti-NKG2D antibody or STI571 pretreatment in human and mouse settings, confirming the role of NKG2D in NK-cell activation. Interestingly, BCR/ABL translocation does not induce DC maturation. BCR/ABL DC have poor allostimulatory capacities, suggesting a reduced risk for GvHD development [81]. Therefore, the clonal BCR/ABL DC displayed the unique and selective capacity to activate NK cells, suggesting that they may participate in the NK-cell control of the disease. However, while STI571 treatment is critical at the early stage of the disease for its direct antileukemic effects, it might be deleterious at later stages because of their effect on NKG2D ligands expression on DC.

Exosomes are small vesicules that are secreted from professional APC such as B cells or dendritic cells. Exosomes will be described in more detail in Chapter 25. DC-derived exosomes (DEX) harbor functional MHC Class I/peptides and MHC Class II/peptides that can induce specific T-cell responses. A phase I clinical trial has been completed in France. Fifteen stage IIIb or IV melanoma patients received four peptide-pulsed DEX vaccines. Since clinical regressions have been observed without any detection of T-cell response, innate effector functions have been assessed on these patients. In 7 out of 14 patients that could be evaluated, NK-cell activation has been detected. *In vitro* studies showed that DEX can enhance CD69 expression on NK-cell patients, and that NKG2D ligands can be found at the surface of DEX. In murine studies, inoculation of DEX derived from immature BM-DC in footpad induce an enhancement of NK-cell number accompanied by an activation of NK cells in draining lymph nodes compared with contralateral LN. The role of NKG2D in DEX-mediated NK-cell activation has been confirmed in this mouse

model using anti-NKG2D neutralizing antibody. The recruitment or proliferation of NK cells is dependent on IL-2-derived T cells because IL-2 neutralizing antibody blocked NK-cell augmentation (Chaput et al., submitted).

24.7 Concluding Remarks

NK cells and DC were identified more than 20 years ago and have been studied almost independently until 1998. DC were then defined as sentinels of the immune system, capable of recognizing "danger" and specialized in the initiation of the adaptive response against danger-causing agents. In addition, DC-dependent activation of $CD8^+$ T cells requires $CD4^+$ T-cell help. Although still valid in many ways, this model has now to face a growing body of evidence showing that (1) not all receptors sensing "danger" or pathogenic agents are expressed by DC; (2) other cells than $CD4^+$ T cells may provide help to DC maturation; (3) DC have the potential to influence both innate and adaptive immunity. In particular, NK cells make functional interactions with DC that can clearly influence induction/regulation of innate and adaptive immune responses. NK activation through NK-cell receptors can promote T-cell responses against tumors, by inducing DC activation. Reciprocally, DC have been found to induce potent NK-cell activation in antiviral responses. IL-12, IL-15, IL-18 and IFN-$\alpha\beta$ production by DC as well as cell–cell contact are critical for the enhancement of NK-cell effector functions.

NK/DC interactions are critical in situations where receptors allowing the recognition of the pathogenic agent are only expressed by one of the two subsets. By extrapolation, one may hypothesize that the expression of receptors allowing early detection of all pathogenic agents is compartmentalized in (innate) immune cell types. In this model, activation of the whole immune system is thus absolutely dependent on cell–cell cross-talk that spreads activation among cells that compose it. At one extreme, DC would be the first cells to be activated by microbes, thanks to their expression of relevant innate sensors (TLR, NOD) and turn on the system. At another extreme, in the case of a tumor that does not cause overt inflammation but does express ligands for activating NK-cell receptors, NK cells would be the first cells to be activated and turn on the system.

It is also intriguing to note the close similarities between the two cell types. Indeed, (1) the development of NK cells and some DC subtypes is selectively dependent on Flt3-L; (2) Some NK-cell markers are expressed on DC (i.e. NKRP1) and vice versa (CD11c): leukemic cells expressing both DC and NK markers and $CD11c^+/DX5^+$ bitypic cells in lymphocytic choriomeningitis virus infection (LCMV) protecting mice against autoimmune diabetes have also been described; (3) DC may have cytotoxic properties or may acquire them in response to stimulation with type I IFN, whereas activated NK cells have recently been identified as potent antigen presenting cells.

Finally, most studies on NK-DC interactions have focused on conditions of microbial and tumoral challenge. It remains to dissect whether NK/DC cross-talk is also involved in autoimmune situations.

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25
Intercellular Communication via Protein Transfer

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25.1
What are Exosomes, and Where do they Come From?

A mediator involved in protein transfer between cells is the exosome. Exosomes are small membrane vesicles formed within multivesicular endosomes, also called multivesicular bodies (MVB), and released in the extracellular space upon fusion of MVB with the cell surface membrane [1–3]. They can be purified from cell-culture supernatant by a series of centrifugation or filtration steps followed by ultracentrifugation to pellet the exosomes. Exosomes are typically between 30–100 nm in diameter (reviewed in [4, 5]). They are secreted by a multitude of cell types, including reticulocytes [6], platelets [7], mast cells [8, 9], B lymphocytes [10, 11], T lymphocytes [12–15], dendritic cells (DC) [11, 16–20], tumor cells [21–24], epithelial cells [25, 26], and trophoblasts [27]. Ample evidence for the presence of exosomes *in vivo* has been documented. They have been demonstrated in various bodily fluids such as serum, urine, malignant effusions, and broncho-alveolar lavage, and at the surface of follicular DC in germinal centers [reviewed in 4]. Furthermore, two lines of research recently converged, as it has been demonstrated that the so-called tumor-derived membrane vesicles, already reported in the early 1980s [Reviewed in 28], are identical with the described tumor-derived exosomes [24].

In general the protein composition of exosomes clearly differs from that of total cell lysates, plasma membrane or apoptotic vesicles, indicating selective sorting events for protein inclusion in exosomes [5, 10, 11, 13, 29]. The common exosome-specific constituents are molecules involved in antigen presentation and co-stimulation, for example major histocompatibility complex (MHC) class I/II and CD86, tetraspanins, annexins, heat shock proteins (hsp), cytoskeletal proteins, raft-associated proteins and glycolipids [Reviewed in 4, 5]. Analysis of exosomes with a different origin revealed also the presence of cell-type specific proteins [8–10, 11, 13, 16, 20, 21, 24, 25, 29–31]. Besides these origin-dependent differences, the maturation or activation state of the exosome-producing cell can influence both the quantity and quality of the exosomes [15, 20]. In several cell types, for example primary DC, epithelial cells, EBV-transformed B cells and tumor cells, the fusion of endo-

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cytic compartments with the plasma membrane, and thus the secretion of exosomes, is considered to be constitutive [10, 20, 21, 24, 25, 32, 33]. However *in vitro* analysis of exosome secretion by immature monocyte-derived DC, indicated that monocytes are poor producers of exosomes during their differentiation process, while immature DC readily secrete exosomes [11, 20]. Further progression from immature DC to mature DC seems to coincide with a reduction in exosome secretion, as LPS-treated mature DC produce less exosomes than immature DC [20, 32]. This indicates that exosome secretion can be regulated during differentiation. Indeed the first description of maturation-dependent exosome secretion was in reticulocytes during the late stages of erythrocyte differentiation [1, 2, 6].

Besides maturation dependent exosome secretion, activation-dependent exosome secretion has been reported. For T cells it has been shown that upon activation via the T-cell receptor (TCR) exosomes containing adhesion molecules, MHC class I and II molecules, and phosphorylated zeta and CD3/TCR, are released more abundantly [13]. Also in mast cells the fusion of late endocytic vesicles with the cell surface is regulated and occurs only after activation [8, 33]. Interestingly, it is not only the quantity of secreted exosomes that differs between immature and mature DC, but also qualitative differences have been observed, as analyzed by protein composition [20]. It is important to note that human and mouse DC-derived exosomes are not completely identical. Exosomes from monocyte-derived human DC, expressing ICAM-1 and CD86 [11], seem to display a phenotype between immature and mature murine DC-derived exosomes [20]. These maturation/activation state-dependent qualitative differences are not only present in DC-derived exosomes, but are also shown in exosome-like vesicles derived from a T-cell clone, which was either anergized or activated *in vitro* [15].

The qualitative differences, represented by the presence of cell-type or activation/maturation state-dependent specific proteins, and the quantitative differences, resulting from maturation/activation state-dependent secretion of exosomes, may define the specific targeting of the exosomes and their biological effect.

25.2
Which Cells are Targets for Exosomes, and how do Exosomes Interact with these Cells?

The physical interaction of exosomes and target cells is an essential step in their ability to perform their physiological function. Exosomes derived from DC, epithelial cells, mast cells, tumor cells, B lymphocytes, and T lymphocytes have been shown to transfer proteins to DC [9, 15, 17–20, 23, 31, 34], B cells [9, 15, 20] or follicular DC [35]. What exactly defines the cellular target of particular exosomes, as well as the interaction mechanism between exosomes and their target, is largely unclear. Different modes of interaction can be envisioned for different cell types, and may relate to specific physiological functions. Exosomes may either merely attach to the cell surface of target cells or fuse with the plasma membrane. Alternatively they can be endocytosed, as also occurs with vesicles derived from apoptotic cells [36–39]. The best characterized example of exosome-mediated protein trans-

fer is the targeting of B-lymphocyte-derived exosomes to the surface of follicular DC in germinal centers [35]. These MHC class II positive B-cell-derived exosomes are present in abundance on the surface of follicular DC, a cell type that neither secretes exosomes nor synthesizes MHC class II molecules [35, 40]. The functionality of adhesion molecules on B-cell-derived exosomes has also been demonstrated in the anchoring of these exosomes to extracellular matrix components, and tumor necrosis factor (TNF) alpha activated fibroblasts [41]. However, until now there is no evidence that exosomes cluster with the plasma membrane of myeloid DC in a manner similar to that described for follicular DC. The mechanism of exosomal interaction with myeloid DC is far less clear. There is ample evidence that exosomes derived from various cellular sources can transfer antigens and functional MHC-peptide complexes to myeloid DC [9, 15–21, 31, 34, 42]. Although it cannot be excluded that exosomes transfer the peptide-MHC complexes directly to the cell surface of myeloid DC, it has been shown that DC have the capacity to internalize exosomes efficiently [9, 21, 22, 43]. The effectiveness of antigen-delivery via exosomes largely depends on the exosomal expression of specific receptors or ligands needed for DC docking [9, 32, 43]. In this respect the presence of hsp in exosomes, and the common receptor for hsp (CD91) on DC could be a pathway for efficient internalization of exosomes [9, 32, 43]. Although it has been suggested that the soluble molecule MFG-E8/lactadherin is an opsonin involved in docking exosomes to target cells [32, 43], this molecule seems not to be essential for addressing exosomes to DC [20]. Other surface molecules such as externalized phosphatidyl serine, CD11a, CD54, and the tetraspanins CD9 and CD81 have also been implicated to play a role in attachment and/or uptake of exosomes by DC [43]. The fact that the ability of bone-marrow-derived DC to capture exosomes decreases with the maturation state, and thereby the endocytic capacity of DC, further supports a role for the endocytic route of exosomes after DC binding [43]. Once internalized and sorted into the endosomal pathway, the DC can process and present antigens derived from exosomes [43]. Such targeting to the endosomal route seems to conflict with the transfer of functional peptide-MHC complexes from exosomes to the plasma membrane of the DC. However, retrograde MHC class II transport from late endosomes to the cell surface, during which endogenous exosomes bearing MHC class II back-fuse with the membrane of MVB has been described [44, 45], and could possibly be a mechanism by which internalized exosomes deliver peptide-MHC complexes to the plasma membrane of the DC.

25.3 What is the Consequence of Exosome Binding or Uptake for the Target Cell?

In studies using DC-derived exosomes or tumor-cell-derived exosomes no evidence was found for DC maturation as a consequence of exosome binding or uptake [21, 34, 46].

Also binding of B-cell-derived exosomes to immature DC did not induce DC maturation [9], while adhesion of these exosomes to fibroblasts readily trigger Ca++ signals [41]. In contrast, mast-cell-derived exosomes induced phenotypical and functional maturation of immature DC, as defined by the upregulation of MHC class II and co-stimulatory molecules and the enhancement of the T-cell stimulatory capacity [9]. Interestingly, although hsp are described to be a common constituent of exosomes, hsp60 and hsc70 are selectively enriched in mast-cell-derived exosomes [9, 21, 29]. The presence of CD91, a common receptor for hsp, on the surface of DC could play a role in the internalization of these exosomes [9], and the functional outcome of this process.

In general B lymphocytes have a poor capacity to activate naïve T cells. Interestingly, exosomes derived from mature DC endow B lymphocytes with the ability to prime naïve T cells, while immature-DC-derived exosomes lack this capacity [20]. Also in the presence of DC, the T-cell activating capacity of mature DC-derived exosomes appeared to be much stronger than of immature DC-derived exosomes [20].

Thus the differential composition of exosomes released by distinct cell types, or under dissimilar conditions, determines the effect on the target cell.

25.4 What is the Physiological Role of Exosomes in the Immune System?

Originally exosomes were thought to function as waste-bins for the elimination of obsolete proteins. This view was based on the fact that during the maturation phase of reticulocytes, secretion of exosomes allows the elimination of proteins that are no longer necessary for the function of differentiated red blood cells [6]. However it remained unclear why, besides the intracellular degradation pathway of fusion of MVB with lysosomes [47], an alternative route of plasma membrane fusion exists [6].

In the immune system activation-dependent, polarized vesicle secretion is a feature shared by a number of different immune cells. NK cells and cytotoxic T cells direct their lytic granules with exquisite timing and precision to the plasma membrane of the target cells [12, 14, 48]. In this process exosomes have an active biological role as messengers to deliver the kiss of death [48]. Activation-dependent secretion of exosomes has also been described for $CD4^+$ T cells and mast cells [8, 13, 15, 33]. The physiological role of these non-lytic vesicles secreted after cell activation, however is less well understood. The same holds true for the more constitutive exosomal secretion by APC, endothelial cells and tumor cells [10, 21, 25, 28, 32, 33].

Exosomes present in the extracellular fluids and circulation can be regarded as an alternative antigen source. It has been reported that high concentrations of exosomes, expressing MHC-peptide complexes and co-stimulation, can activate *in vitro* T-cell clones and lines very weakly [10, 42], but fail to stimulate naïve T cells [18, 22]. However, in the presence of DC the T-cell stimulatory capacity of these exosomes was highly increased [18–20, 34, 42]. This led to the hypothesis that exo-

somes transfer proteins to APC in a functional manner [9, 17–20, 23, 31, 34, 42, 43]. Indeed tumor-peptide loaded DC-derived exosomes can strongly stimulate tumor-specific cytotoxic T-cell responses after *in vivo* administration [16]. These promising findings have already resulted in a phase I clinical trial in which metastatic melanoma patients were immunized with autologous DC-derived exosomes pulsed with MAGE 3 peptides [49]. The physiological role of exosomal transfer of MHC-peptide complexes between DC could be the amplification of the T-cell response as a result of the increase of the number of antigen presenting cells (APC) bearing the specific peptide-MHC complexes [16, 18, 34]. Alternatively, exosomes could be a vector for the transfer of antigens from migrating DC, such as Langerhans cells emigrating from infected skin to draining lymph nodes, to resident lymph node DC, such as CD8+ DC [50]. Not only DC-derived exosomes are transferred to other DC, also exosomes derived from mast cells, epithelial cells, tumor cells and T cells have been shown to target DC [9, 15, 22, 26]. It is possible that exosomes play a role in the immune surveillance by transferring cell type-, activation/maturation state-, and micro-environmental specific snapshots to DC. As such, exosomes are not exclusively linked to T-cell stimulatory properties but in fact can also induce tolerance. Several studies have shown that anergic regulatory T cells can modulate the APC function by inducing a tolerogenic phenotype after cell–cell contact [15, 51–54]. Recently, it has been found that both activated anergic and effector T cells donate MHC-peptide complexes to APC [15]. However, only APC that acquired MHC-peptide complexes from regulatory-T-cell-derived exosomes became tolerogenic, while effector-T-cell-derived vesicles endowed the APC with T-cell stimulatory properties [15]. This led to the hypothesis that the exosomal transfer of an as yet not defined anergic-T-cell-derived molecule endowed the APC with tolerogenic properties. In this respect the identification of molecules associated with immune regulation in exosomal preparations of several cell types is a major future challenge.

For tumor-cell-derived exosomes it has been found that transfer of tumor antigens to DC can result in crosspresentation and specific activation of cytotoxic T cells [21, 22]. However, tumor cells have also developed strategies to circumvent immune recognition, such as the ectopic expression of the human leukocyte antigen (HLA) class I molecule HLA-G, interacting with inhibitory receptors on immune cells [23]. In melanoma-derived exosomes the presence of HLA-G could be demonstrated, which may result in the inhibition rather than the enhancement of the T-cell stimulatory capacity of targeted DC, thereby allowing the tumor to escape immune surveillance [23]. Another example is the presence of bioactive FasL in exosomes secreted by tumor cells [24, 55], or by T-cell leukemia and normal human T-cell blasts after mitogenic stimulation [14]. FasL-containing exosomes could be involved in the induction of autocrine or paracrine cell death during immune regulation. Such immune regulatory function of exosome-associated FasL has also been suggested to play a role in the tolerance for paternal antigens during pregnancy, as trophoblasts appeared to secrete FasL-containg exosomes [27]. Tumor cells may exploit this mechanism and may shed FasL-containing exosomes to subvert the immune system, and thereby escape immune surveillance [24, 56].

Tab. 25.1 Exosomal transfer of membrane proteins between (immune) cells.

Source of exosomes	*Target cell*	*Reference*
DC	DC	16–20, 34, 43, 46
Tumor cell	DC	21, 22
Epithelial cell	DC	26, 31
Mast cell	DC, B lymphocyte	9
CD4 T lymphocyte	DC, B lymphocyte	15
DC	B lymphocyte	20
B lymphocyte	Follicular DC	35

Other examples of tolerance induction after administration of exosomes have been reported in a heart transplantation model in which injection of donor DC-derived exosomes, but not recipient DC-derived exosomes, before transplantation resulted in tolerance rather than immunity [17]. Also injection of exosomes released by intestinal epithelial cells of antigen fed rat resulted in antigen-specific tolerance in naïve recipients [26]. Although most studies focused on the exosomal protein transfer to DC, exosomes also target other cell types, for example T cells [57]. Since T cell–T cell presentation has been shown to be very efficient in anergy induction [reviewed in 58], exosomal protein transfer to T cells may also be involved in tolerance induction.

In summary, exosomes can serve as intercellular messengers for the transfer of proteins between a wide variety of cells (Table 25.1). The outcome of the immune response induced by exosomal protein transfer largely depends on the cellular origin of the exosomes and their target cell.

25.5
Cell–Cell Contact-dependent Transfer of Membrane Proteins

Besides exosome-mediated protein transfer, a cell–cell contact-dependent manner of intercellular transfer has been observed as well. The transfer of membrane fragments across immunological synapses [59] emerges as a common route of communication between immune cells. For a long time protein acquisition by immune cells at the immunological synapse has been regarded as a unidirectional process from target cell or APC to effector cell. For T cells it has been shown that they acquire not only MHC class I and II peptide complexes and lipids from APC during T cell-APC interaction, but also incorporate other membrane associated proteins such as, Ig, CD80, CD86, OX40L, CD4, IL2-R, and ICAM-1 [60–69]. After specific receptor–ligand interactions have established cell–cell contact, such as CD28-CD80/CD86 or TCR-peptide-MHC, a wide variety of surface molecules from APC can be absorbed by antigen-experienced and naïve CD8 and CD4 T cells [60, 61, 65, 70]. Both resting and activated T cells can absorb molecules from APC, albeit activated T cells are more efficient [60, 69]. Importantly, this phenomenon is not an *in*

vitro artefact since it also occurs *in vivo* [60, 69]. Once the T cell is activated, TCR or CD28 ligation is no longer required for the direct capture of MHC-peptide complexes [64, 69, 71]. Activated T cells acquire MHC molecules not only from syngeneic APC, but also from allogeneic and xenogeneic APC [64, 71, 72]. Furthermore, activated, but not resting, CD4 T cells can acquire a variety of molecules in an antigen or ligand nonspecific manner after cell–cell contact with endothelial cells, DC, and monocytes [66, 71, 73]. This suggests that TCR ligation is required for T-cell activation, but is thereafter not strictly needed for acquisition of molecules.

Besides the acquisition of APC-derived molecules several immune cells have been shown to absorb molecules from their target cells. Cytotoxic T cells can rapidly capture membrane fragments from target cells in a TCR signaling-dependent fashion [62, 63]. Since target cells did not acquire CD8 from cytotoxic T cells, it has been assumed that this is a unidirectional process [63]. Also B cells can absorb membrane proteins from target cells. After immune synapse formation, the B cell receptor mediates the efficient capture of integral membrane proteins expressed on target cells [74]. NK cells rapidly acquire MHC class I molecules and viral receptors from target cells after formation of an inhibitory immune synapse between NK cells and target cells [75–77]. However, in the inhibitory immune synapse cell surface proteins are not only transferred from target cell to NK cell, but also vice versa from NK cell to target cell [78]. Although in many reports it is assumed that protein transfer in the APC-T cell immunological synapse is a unidirectional process from APC to T cell, more and more evidence is accumulating that protein transfer is in fact bidirectional [15, 71, 79, 80]. In this respect the description of homotypic synapses between leukemia cells, and the bidirectional transfer of membrane fragments across these synapses is also worth mentioning [81].

Besides the fact that DC can readily capture antigen from dead and apoptotic cells for presentation to MHC class I restricted CTL, they can also acquire cell surface molecules from viable endothelial cells, macrophages, B cells, activated T cells, and other both mature and immature DC in a cell–cell contact-dependent manner [15, 79, 80, 82–85]. Whether the maturation state of the DC influences the efficiency of membrane molecule absorption from other cells is still a controversial issue [79, 80, 82]. Notably macrophages, well known for their excellent phagocytic capacity, appeared to be rather inefficient in acquiring membrane molecules from other cells [80].

It has been assumed that resting immune cells do not acquire membrane fragments from cells transiently encountered along nonproductive interactions. However, it appeared that organized immunological synapses are also formed between mature DC and naïve T cells in the absence of antigen, and even in the absence of MHC molecules, and that these antigen-independent synapses allow the transmission of signals [86, 87]. Whether in these antigen-independent synapses, intercellular protein transfer also ensues, remains to be seen.

Although the precise requirements for intercellular protein exchange, and the transfer direction are not fully understood, the phenomenon of swapping membrane molecules has been acknowledged to occur between a wide variety of immune cells (Table 25.2).

Tab. 25.2 Protein transfer between (immune) cells during cell–cell contact.

Protein donor	*Protein acceptor*	*Reference*
DC	CD8 T lymphocyte	60, 68, 69
"Model"APC[1]	CD8 T lymphocyte	60–62, 69
DC	CD4 T lymphocyte	60, 65, 69
"model"APC	CD4 T lymphocyte	60, 65, 66, 69
Target cell	CD8 T lymphocyte	63
Endothelial cell	CD4 T lymphocyte	66, 73
Endothelial cell	DC	82
T lymphocyte	DC	79
Melanoma cell-line	DC	79
Adenocarcinoma cell-line	DC	85
DC	DC	82, 85
Target cell	NK cell	75–78
NK cell	Target cell	78
Leukemia cell	Leukemia cell	81

[1] Model APC; Drosophila cells [60, 61, 69], DC cell line [61], RMA-S [61], Transfected RMA and 3T3 [62], Fibroblast cell lines [65], COS-1 cell lines [65, 66], A20 [65], MC38 cell lines [65], Jurkat transfectants [66], SVT2 transfectants [66].

25.6
How are Membrane Proteins Transferred Between Immune Cells, and What is their Fate?

The mechanism of direct cell–cell contact-dependent transfer of membrane molecules is largely unknown. In several studies it has been shown that both proteins and lipid markers are transferred, suggesting that membrane fractions rather than soluble proteins are exchanged [15, 62–64, 73, 79, 80]. Besides transfer via exosomes, it has been suggested that other vesicles, for example those formed from extracted plasma membrane fragments, are involved in the synaptic protein transfer process [74, 80]. Alternatively, membrane fusion leading to the formation of membrane bridges between cytotoxic T lymphocytes and target cells or between APC and T cells [63, 70], and the formation of membrane nanotubes connecting multiple cells [63, 88], have been demonstrated and implicated as means for protein transfer. Overall, intercellular protein transfer is a complex process during which several different mechanisms may run in parallel (Fig. 25.1).

The process of active protein transfer triggered by receptor signaling, which occurs rapidly after conjugate formation between cells, has been called "trogocytosis" (Troxis is Greek noun derivative of 'trogo' the ancient Greek verb meaning 'to eat, chew, gnaw') [89]. Trogocytosis (troxis necrosis) originally referred to the gradual disappearance of hepatocytes as a result of lymphocyte–hepatocyte binding and internalization of liver surface molecules by the lymphocyte during hepatitis [90]. Several reports describe APC-derived MHC molecules, which are rapidly absorbed by T cells, being co-internalized with the TCR within several hours through endo-

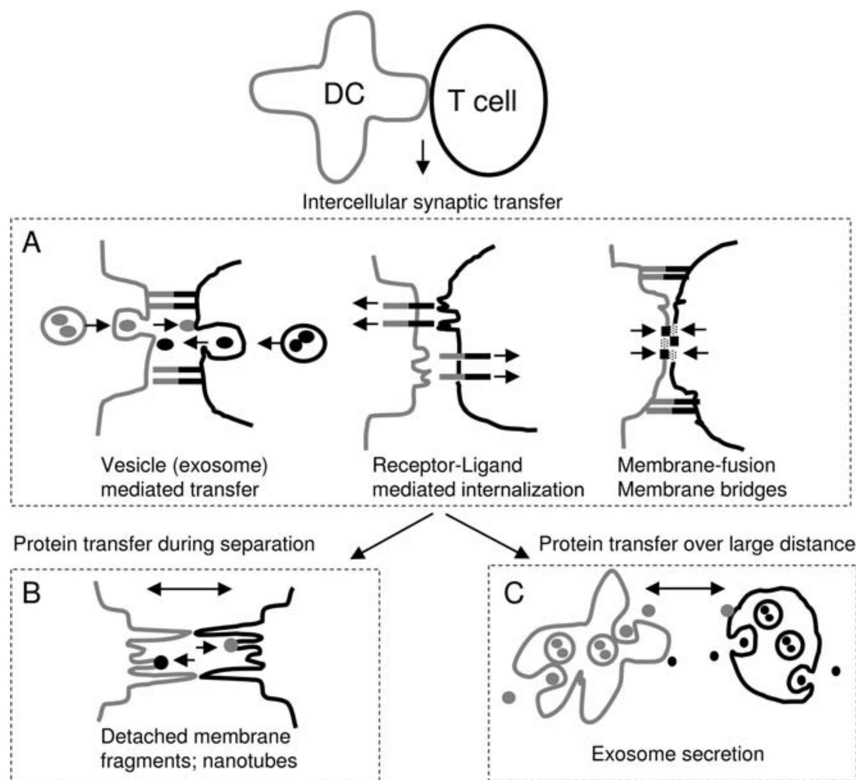

Fig. 25.1 Intercellular transfer of membrane proteins: A give-and-take relationship. Schematic representation of possible mechanisms involved in intercellular protein transfer. (A) During the intimate contact between a DC and T cell, protein exchange can occur in the immunological synapse. (B) During the separation of the DC and T-cell membrane fragments can be exchanged as a consequence of mechanical traction. C) After activation, the DC and T cell secrete exosomes, which can act as vector for protein transfer over a larger distance.

cytosis, and being localized in endosomes and lysosomes [60, 61]. However, receptor-mediated internalization is probably only part of the story, as it has been demonstrated that after immune synapse formation an enormous quantity and a wide variety of molecules can be absorbed [60, 62]. For resting T cells the requirement for cell–cell contact appeared to be more stringent than for activated T cells, which also show absorption of proteins in a trans-well system [69]. Once activated, T cells can absorb molecules in the absence of specific ligands [64, 66, 71]. Entire proteins are transferred and stabilized in the recipient cell membrane, often seen as discrete punctate formation, in a functional manner [60–63, 65, 66, 69, 70]. The role of the cytoskeleton in the absorption process also differs between resting and activated T cells, because resting T cells appeared to be more dependent on the actin cytoskeleton for both TCR- and CD28-mediated protein absorption [69]. Interestingly, this dependence was lost when the specific MHC-peptide concentration on the APC

was increased [69]. These findings illustrate that the requirements for protein absorption by T cells are rather complex, and depend on the activation state (resting versus activated T cells), maturation state (naïve versus memory T cells), the molecules involved (TCR, CD28 etc.), and the concentration of ligand on the donor cell.

Similar to protein acquisition by T cells, protein absorption by DC can either result in internalization, processing and presentation of the acquired antigens, or in the functional integration of intact acquired protein complexes in the plasma membrane [79, 80, 82, 85]. Recently, a new route of intercellular peptide exchange via gap junction channels between cells has also been described, via which peptides are transferred from the cytoplasm of one cell into the cytoplasm of its neighbor [91]. Also monocyte-derived DC are capable of using this route for intercellular transfer of small peptides [91].

25.7 What is the Physiological Role of Membrane Protein Swapping in the Immune System?

Although the physiological role of intercellular protein exchange is largely unknown, several interesting hypotheses have been postulated. It has been speculated that absorption of membrane molecules in the immunological synapse is needed for the dissociation of the two cells forming the synapse. Indeed the formation of tight clusters between T cells and APC raises the question of how these cells can subsequently disengage. The rapid absorption and internalization of ligands at the contact site could play a role in T cell–APC dissociation, allowing the activated T cell to move from one APC to another [60].

Alternatively, the efficient MHC-peptide stripping of APC by high affinity T cells may provide a mechanism of T-cell competition for specific MHC-peptide ligands. This could play a role in affinity maturation of T-cell responses, giving the biggest advantage to high affinity T cells [68, 80]. The absorption and internalization of MHC-peptide complexes might also be involved in limiting the risk of over-stimulation of the antigen specific T-cell population.

Depending on the fate of the transferred molecules, intercellular protein exchange between immune cells may also result in the inheritance of functional molecules from conjugating cells, which are not transcribed by the recipient cell. This may influence not only the phenotype, but also the function of the recipient cell. In this respect trogocytosis should be regarded as a vector for intercellular communication. The functional acquisition of APC or target cell-derived MHC-peptide complexes by cytotoxic T cells may play an active role in immune regulation by the induction of fratricide. During this process T cells become sensitive to lysis by neighboring cytotoxic T cells specific for the same ligand [61–63]. This process could be involved in the elimination of cytotoxic T cells that have interacted with many targets, and as such play a role in the down-regulation of the immune response. Also functional MHC class II molecules can be acquired from professional APC by activated T cells [60, 61, 63, 64, 68]. Since MHC class II expression

on T cells can result in the induction of anergy [Reviewed in 58], it can be envisaged that acquired MHC class II-peptide complexes presented on activated T cells may contribute to immune regulation [64, 65]. On the other hand T cells not only acquire MHC-peptide complexes from APC, but can also absorb co-stimulatory molecules, for example CD80 or OX40L [65, 66]. Such T cells may act as professional APC, and as such give rise to an enhanced immune response [65, 66].

DC can present exogenous antigens derived from the extracellular milieu or captured from neighboring cells via the MHC class I antigen presentation route, a process called cross-presentation [92]. The capacity of DC to acquire intact MHC-peptide complexes from viable cells, and the functional presentation of these complexes on the DC surface, can be regarded as a pathway for cross-presentation [15, 79, 80, 82–85].

Whether the cross-presentation ultimately results in T-cell tolerance or immunity depends on the context in which the protein transfer takes place, meaning the composition of transferred proteins and the resulting effect on DC maturation and activation. Both the quantity and quality of membrane protein swapping during T cell–APC contact is affected by the strength of the interaction, and the activation and maturation state of the T cell and APC. As such intercellular membrane protein transfer between T cells and APC seems to represent a cell–cell contact-dependent manner to regulate immune responses.

25.8
Concluding Remarks

It is challenging to postulate that intercellular protein transfer plays a significant role in the regulation of the immune response. The further dissection of immunogenic versus tolerogenic signals delivered by exosomes or membrane patches is an important future challenge. Insights into these different aspects of intercellular protein transfer may offer novel opportunities for the development of immunotherapeutic strategies not only for the treatment of cancer, but also for prevention of allograft rejection and autoimmune diseases.

Abbreviations

DC	dendritic cell
NK	natural killer cell
MVB	multivesicular body
MHC	major histocompatibility complex
hsp	heat shock protein
TCR	T-cell receptor
TNF	tumor necrosis factor
APC	antigen presenting cell
HLA	human leukocyte antigen

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X
Tolerogenic Dendritic Cells

26
Differentiation Stages and Subsets of Tolerogenic Dendritic Cells

Manfred B. Lutz

26.1
Introductory Remarks

Dendritic cells (DC) represent sentinel cells integrated in or residing close to epithelia and thus standing first in line to face invading pathogens. They are major players of the immune system to distinguish self from non-self [1, 2]. Equipped with pathogen recognition receptors, such as the Toll-like receptors (TLR), DC can recognize evolutionarily conserved microbial structures and transmit "danger" signals which lead to DC maturation and migration to the draining lymph node [3, 4]. Although DC are located in almost all body tissues, their frequency is higher in epithelia and also in secondary lymphoid organs such as the thymus, spleen and lymph nodes. However, the high frequency of DC in the secondary lymphoid organs during the steady state may point to their permanent role in tolerance induction, besides their central role for adaptive immune responses after activation. While it was accepted quite early that antigen presentation by DC in the thymus is involved in central tolerance, there is recently accumulating evidence that DC of the spleen and lymph nodes regulate peripheral tolerance. There is considerable hope that such tolerogenic DC might enter clinical application in transplantation, autoimmunity and allergy/asthma [5–11]. In this chapter the tolerogenic capacity and mechanisms of different DC subsets and differentiation stages of DC will be reviewed (Fig. 26.1).

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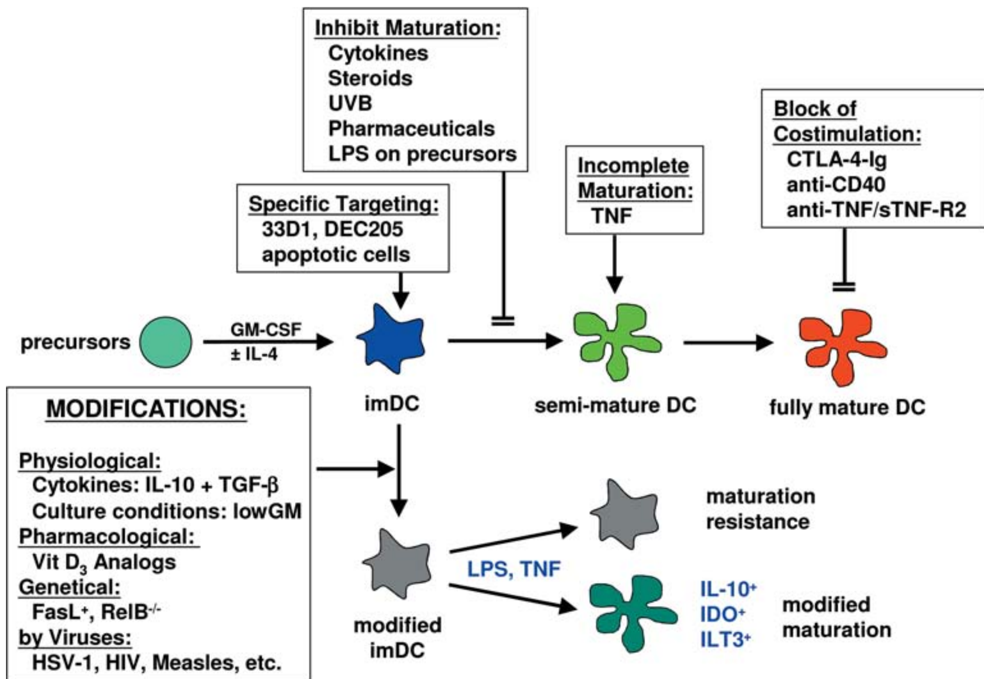

Fig. 26.1 Methods of *in vitro* generation or *in vivo* targeting of tolerogenic DC. Many methods of generating tolerogenic DC are focused around immature DC. They can be targeted specifically *in vivo* by delivery of apoptotic material or targeting to specific surface receptors. Spontaneous maturation of *in vitro* generated DC can be achieved by addition of maturation inhibitors. Inhibition of *in vivo* maturation is attempted by inhibiting co-stimulation of already matured DC. Incompletely matured DC (semi-mature DC) can still be tolerogenic. Due to the problem of maintaining stable immature DC, DC modifications are desired that result in maturation-resistant immature DC or modified/alternatively matured DC expressing inhibitory surface receptors (IDO, ILT-3) or cytokines (IL-10).

26.2
Mechanisms of T-cell Tolerance Induction

The mechanisms of T-cell tolerance have been categorized into five distinct phenomena: ignorance, anergy, deletion, immune deviation, and regulation/suppression. They have been observed by using DC *in vitro* and in mouse models *in vivo* [12] and may help to explain the “behavior” of T cells when stimulated under highly controlled conditions but, as demonstrated below, the maintenance of tolerance *in vivo* appears more complex and may not be attributed exclusively to the one exclusive mechanism.

26.2.1 Ignorance

When antigens expressed in peripheral organs do not reach the blood or secondary lymphoid organs to be presented or crosspresented on MHC I and II molecules, T cells specific for these antigens remain ignorant. Conversely, circulating naïve T cells will not be able to extravasate and survey the tissues. Although ignorance has been postulated as a theoretical mechanism to maintain tolerance, it is difficult to demonstrate *in vivo* whether an antigen is not presented at all or presented at very low levels so that specific effects of T cells are below the detection limit. For obvious reasons there is no role for DC in ignorance as non-presenting cells.

26.2.2 Anergy

T-cell anergy, or hyporesponsiveness, was first demonstrated with T-cell clones *in vitro* [13]. The theory of clonal T-cell anergy induction proposes that a TCR signal (signal 1) is provided in the absence of a co-stimulatory signal through the CD28 molecule (signal 2). However, both signals are required for IL-2 gene activation and clonal T-cell expansion [14].

The TCR signal alone induces first, a Ca^{2+} influx resulting in NF-AT activation, which then trigger a series of signaling events, including GRAIL activation which inhibits IL-2 production by the anergic cells [15–17] and second, CTLA-4 upregulation. A second encounter of such partially preactivated T cells with APC competent in B7-1/B7-2 co-stimulation will now preferentially trigger the CTLA-4 molecule because its affinity is 10-fold higher than CD28 [18, 19]. CTLA-4 signals activate the cell cycle inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ [20, 21] and potentially other factors [22], and thus prevents cell cycle entry and IL-2 production. Thus, partial T-cell stimulation leads to their suboptimal activation, resulting in anergy, characterized by TCR hyporesponsiveness, block of cell cycle entry and impaired IL-2 production [23]. Nevertheless, in most anergy settings the T cells remain responsive to high doses of IL-2 alone without further stimulation of the TCR and CD28 which is in contrast to reg T (see below).

Many of the T cells engaged in this way might die from apoptosis while the surviving T cells persist alive for at least one month in mice remaining in an anergic state. During this time period anergic T cells may acquire regulatory functions on other effector T cells and DC [24, 25].

26.2.3 Deletion

The most clear-cut form of T-cell tolerance is the complete deletion of T cells with unwanted specificity. This form of T-cell elimination has been described in different settings. Deletion of T cells can occur in the absence of co-stimulation [26, 27], by the lack of growth factors [28] or space [29], i.e. as a result of suboptimal stimu-

lation. In contrast, deletion commonly occurs as a result of optimal T-cell priming by activation-induced cell death (AICD) in order to limit or terminate T-cell responses of activated T cells through CD95/CD95L dependent mechanisms [30–33].

Especially for $CD8^+$ CTL responses, the deletional pathway is well documented [34–36]. Presentation of exogenous antigens on MHC I molecules occurs via cross-presentation and can also lead to deletional tolerance [37].

26.2.4
Immune Deviation

T helper cell differentiation can occur two major pathways. The Th1 differentiation gives rise to predominantly IFN-γ producing T cells while Th2 cells produce IL-4, IL-5 and IL-13. As the Th1 cytokines can promote $CD8^+$ CTL responses, this arm of the adaptive T helper cell activity has been called "cellular immune response". In contrast, the Th2 cytokine IL-4 was the first to be identified as promoting the isotype switch of B cells to IgE, and therefore the Th2 help has been called a "humoral immune response". This dichotomy is not rigidly applied since Th1 cytokines can also support certain isotype switches (IFN-γ to IgG2a in the mouse) [38] and the activity of macrophages and NK cells partially depends on antibody binding to Fc receptors for microbial recognition or antibody-dependent cellular cytotoxicity (ADCC).

The phenomenon of immune deviation is based on the observation that T helper cell priming can be polarized towards Th1 in the presence of IFN-γ and blocking of IL-4, while Th2 polarization is driven by IL-4 and blocking of IL-12 and/or IFN-γ [39]. If an immune response against a microbe requires Th1 immunity to resolve the infection, such as *Leishmania major* in C57BL/6 mice, the immune response is dramatically impaired if it is polarized towards the "wrong" Th2 arm as observed in BALB/c mice. As a fatal result BALB/c mice succumb to the infection [40]. This indicates that a given pool of naïve T cells with specificity for a certain antigen can be differentiated in either the Th1 or the Th2 direction, and all subsequent immunizations for the same antigen will then also follow the preset type of memory immune response.

26.2.5
The Concept of "Immune Balance"

If a harmless antigen enters the body, it is conceivable that a nonfatal type of immune reactivity may be induced. This may be especially applicable for allergies. For example, in nonallergic individuals an immunoreactivity to the Bet v 1 allergen can be observed, but the responsive T-cell clones produce predominantly IFN-γ and little IL-4 and the serum contains substantial IgG but no detectable IgE. In this way an involvement of mast cells in the immune response is avoided [41]. Thus, only if the "wrong" type of immune response is initiated, may diseases such as allergies and asthma develop. This concept of permanent immune deviation to harmless antigens/allergens has also been called "immune balance" [42, 43].

26.2.6
Regulation/suppression

Regulatory T cells (reg T) represent specialized effector T cells which are able to downregulate immune responses. All reg T cells have in common that they require TCR stimulation to activate their suppressive mechanisms, which then, however, can act antigen-unspecifically and also provide bystander suppression. Two distinct classes can be distinguished: (a) the thymus-derived naturally-occurring $CD4^+$ $CD25^+$ $Foxp3^+$ reg T cells, which constantly express the IL-2Rα chain (CD25) and mainly regulate through cell contact-dependent mechanisms [44, 45]; (b) several types of peripherally induced reg T such as the Tr1 which mainly regulate through secretion of IL-10 [46]. Also Th3 cells have been described which mainly secrete TGF-β and thereby act suppressively [47]. In addition there are also $CD4^-CD8^-$ reg T [48, 49] and $CD8^+CD28^-$ reg T subsets [50] with dramatic suppressor functions.

The role of reg T during the steady state *in vivo* is unclear. The induction of both Tr1 and $CD4^+$ $CD25^+$ reg T requires co-stimulation through CD28 [51, 52]. This would indicate that immature DC in the steady state cannot be responsible for their induction because they are largely incompetent for co-stimulation. In fact, in healthy individuals all $CD4^+CD25^+$ reg T cells are in a resting state, i.e. negative for activation markers such as CD69. Also experiments comparing immature and mature DC in mice clearly showed that mature DC were superior in the activation and expansion of $CD4^+CD25^+$ reg T cells [53, 54].

However, human immature MoDC have been shown to stimulate the occurrence of IL-10-producing Tr1 *in vitro* [55]. Immature MoDC already express certain amounts of co-stimulatory molecules CD80/CD86. Together, this might indicate that low levels of TCR stimulation plus low levels of co-stimulation might be sufficient for reg T activation.

26.2.7
Combinations

When the fate of antigen-specific T cells is followed *in vivo* and the analysis is not restricted to the determination as to whether a certain tolerance mechanism occurs or not, a combination of several tolerance mechanisms mostly seems to occur.

Anergy and cytokine-mediated suppression can occur simultaneously in models of tolerance against superantigens in mice [56] and anergic T cells can turn into suppressing/regulatory T cells *in vivo* [57, 58] and as shown for human T cells *in vitro* [59].

Many models of *in vivo* tolerance induction observed an initial expansion of the antigen-specific T cells after antigen application followed by their deletion after a couple of days and finally the few surviving T cells showed signs of anergy. This has been studied for the nonimmunogenic i.v. injection of superantigens [58, 60] or OVA protein [61–63].

Together, the kinetics of tolerization or immunization of T cells *in vivo* seem quite similar but occur at different levels of intensity (Fig. 26.2). Parameters direct-

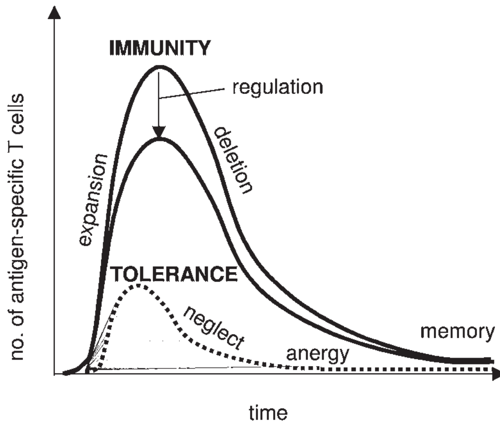

Fig. 26.2 Quantitative and kinetic view of T-cell immunity and tolerance. Induction of T-cell immunity, e.g. by vaccination, is accompanied by the clonal expansion of the antigen-specific T cells, followed by the decline of cell numbers (deletion) and resulting in the maintenance of an antigen-specific memory T-cell pool. All these processes can be suppressed, e.g. by regulatory T cells. If tolerance is induced, e.g. by systemic application of an antigen, the series of events remains similar to immunization, but with lower numbers due to the suboptimal activation levels of the individual T cells. The expanded T cells decline here rather due to death by neglect and result in long-lived anergic T cells.

ing tolerance or immunity might therefore rather depend on the dose, duration and timing of antigen presentation [29, 64–66]. Many of these parameters can be directed by different types of APC but also by different maturation stages of DC.

26.3 Tolerogenic DC Subsets *in vivo*

One major question regarding tolerogenic DC is whether certain subsets of DC exist with an intrinsic tolerogenic potential as opposed to the sensor-DC that upon activation become immunogenic DC. The current literature suggests that environmental signals can either inhibit DC maturation or modify DC so that they convert to tolerogenic APC.

26.3.1 Thymic DC

DC resident within the thymus are involved in thymocyte selection by presenting antigens from the circulation [67]. Thymic DC present these antigens for longer time periods as compared with DC of other organs [68]. A closer analysis of thymic DC revealed that they are shortlived and predominantly of the MHC II^+, $CD11c^+$, $CD205^+$, $CD8\alpha^+$ subtype [69, 70]. So the tolerogenic nature of antigen presentation

by thymic DC does not seem to be intrinsically different from that of the spleen. Rather, the responses of immature thymocytes and mature T cells seem to determine different outcomes [71]. Intrathymic injection of isolated thymic or splenic DC has been shown to induce T-cell anergy to Mls antigens [72].

26.3.2
DC in Lymph Nodes and Spleen

DC reside as interdigitating cells in the spleen and lymph nodes even in the steady state and not only after infections. There they can be characterized either by their immature or partially mature state [73] or as phenotypically distinct and functionally specialized subsets (see Chapter 12).

DC within spleen and lymph nodes abundantly present self-antigens in the steady state as measured by the self-peptide/MHC II complex recognizing antibody Y-Ae [74]. DC from Peyer's patches constitutively produce IL-10 and after isolation induce IL-4 and IL-10 releasing T cells [75]. When CD8 T cells were followed in inducible transgenic mice where both DC and antigen-reactive T cells appear at a physiological frequency, strong tolerance is induced against a *de novo* induced antigen [76].

Similarly to the DC1/DC2 paradigm for the induction of respective Th1 or Th2 immunity, it is now a matter of debate whether certain intrinsically tolerogenic DC subsets exist or whether all known DC subsets can exert tolerogenic function, depending on their environmental instruction. One source of environmental signals for DC might be derived from low level and locally restricted apoptotic cells which occur continuously during tissue remodeling. In the mouse spleen two major DC subsets have been characterized quite well. One is the classical myeloid $CD8\alpha^-$ DC and the other was initially termed "lymphoid" DC characterized by their $CD8\alpha$ expression. *In vitro* and *in vivo* apoptotic cell uptake could be observed by $CD8\alpha^+$ DC which crosspresented the apoptotic material and induced initial $CD8^+$ T-cell expansion followed by deletion [12, 77] or induced $CD8^+$ T-cell unresponsiveness [78]. As the $CD8\alpha$ expression largely overlaps with the CD205 expression, the targeting of antigens to this specific molecule may also be attributed to this DC subset [79, 80]. It is of note that in most of the experiments used to analyze lymph node DC, resident versus immigrating DC from the draining tissues have not been analyzed separately.

26.3.3
Migratory DC from Peripheral Organs

Peripheral lymph nodes draining the skin also contain mature DC during the steady state. These DC are MHC II^{high}, B7-2^{high}, $CD40^{high}$ DC and clearly represent epidermal LC and dermal DC which have migrated to the lymph node [73]. Their steady-state migration is dependent on CCR7 expression [81]. Such migratory DC are not induced by any pathogenic or normal skin flora as they can be also detected in germ-free mice (Kanazawa and Lutz, unpublished observations).

In peripheral lymph nodes draining various organs, the transport of apoptotic material during the steady state has been demonstrated. The earliest reports show the transport of melanocytes by epidermal LC [82] which has later been confirmed using a transgenic melanocytosis model [83]. Cannulation of the pseudo-afferent lymphatics in the rat revealed two populations of migrating DC, with one transporting self-antigens from the intestine to the mesenteric lymph node [84]. Similarly, inhaled antigen is transported by DC to the lung-draining lymph node and there induced Tr1 cells [52]. This mechanism might be exploited by microorganisms for immune escape as demonstrated for *Bordetella pertussis* [85]. Two more groups show the presentation of cell-associated self-antigens in the lymph nodes draining the stomach [86] or pancreas [87]. Although this presentation might indicate the self-antigen transport by migratory DC, this has not been demonstrated.

26.3.4 Plasmacytoid DC

Initially the plasmacytoid DC (pDC) have been termed DC2 for their capacity to induce immune deviation by promoting Th2 responses and thereby inhibiting Th1 cells [88, 89]. However in the mouse, the contrary direction of deviation has been demonstrated by pDC. Murine Th2 cell mediated asthma could be prevented by pDC but not myeloid BM-DC [90]. *In vivo*, $B220^+$ pDC can be detected in the thymus, lymph nodes and the spleen in an immature/resting stage where they might induce $CD4^+$ T cells with regulatory capacity [91], T-cell anergy [92] or $CD8^+$ IL-10-producing reg T [93]. A distinct $B220^-$ $Gr\text{-}1^-$ $CD11c^{low}$ $CD45RB^{hi}$ DC population with plasmacytoid morphology was found in murine spleens to be potent producers of IL-10 [94]. Such IL-10 producing pDC might develop under tolerogenic conditions *in vivo* by the influence of factors derived from splenic stroma cells [95]. Triggering of the CD200 receptor on pDC induces the release of indoleamine-2,3-dioxigenase (IDO) and initiates tryptophan catabolism which impairs T-cell functions [96].

26.4 DC Precursors

Besides the tolerogenic capacity of differentiated DC, there is accumulating evidence that DC precursors might also bear tolerogenic capacity. During the culture period of 8–10 days for BM-DC generation with GM-CSF according to a standard protocol [97], the DC precursors go through a stage around day 3–4 where they transiently acquire suppressive capacities (Table 26.1) [98]. Such DC precursors have been described as myeloid suppressor cells (MSC) with major influence on Th1 cells in *Candida* infection [99] and $CD8^+$ CTL responses in murine tumor models [100–102]. Human MSC seem to influence antitumor immune responses in patients with head-and-neck cancer [103–106] but they are poorly characterized.

Tab. 26.1 Characteristics of murine suppressive DC precursors (i.e. Myeloid Suppressor Cells, MSC).

Morphology	non-adherent, irregular round cell shape, ring-shaped nucleus
Antigen uptake	$CD14^{-}$, $TLR4^{-}$, $Fc\gamma R$ $II\&III^{+}$, $CD205^{+/-}$,
Antigen presentation	MHC I^{+}, MHC II^{-}, $CD1d^{+}$
Co-stimulation	$CD80^{+}$, $CD86^{+/-}$, $CD40^{-}$, $CD54^{+}$
Development	$CD34^{-}$, $CD31^{+}$, ER-$MP58^{+}$, $CD13^{-}$, Gr-1^{low} (Ly-$6C^{+}$, Ly-6G ^{low}), CD11c–
Lineage	$CD4^{-}$, $CD8\alpha^{-}$, $B220^{-}$, $NK1.1^{-}$, $DX5^{-}$, $asialoGM1^{+}$, $F4/80^{+}$, **$CD11b^{+}$**, $MOMA1^{-}$, $33D1^{-}$, $CD169^{-}$
Homing	$CD62L^{+}$, $CCR7^{-}$, $CCR5^{-}$
Activation requirement	IFN-γ
Suppressive mechanisms	cell-contact required, NO release
Disease implication	suppression of antitumor CTL immunity, NOD

26.5 Immature DC

26.5.1 Tissue Resident DC

The prototype of an immature DC *in vivo* is the epidermal Langerhans cell in a healthy individual. In the steady state LC show a stellate morphology and together form a meshwork structure at a high cell density (1000 LC mm^{-2}) to sense for pathogens. LC express very little MHC II on their surface and B7 molecules are absent [107]. In this respect they are even less mature than the immature DC residing in the spleen and lymph nodes [108]. Also all other organs contain about 1% tissue-resident, immature DC but the percentages are highest in epithelial and secondary lymphoid tissues (2–4%) [109].

26.5.2 Induction of T-cell Anergy by Immature DC

Immature DC are characterized by their high capacity to endocytose antigens but low levels of MHC I and II and co-stimulatory molecule expression on the cell surface [110] and therby fulfill the criteria for anergy induction.

Intracellularly, immature DC accumulate large amounts of MHC II molecules within different types of endosomal compartments where antigen loading can take place [111]. Immature DC also continuously endocytose antigens or apoptotic material from their environment [112, 113] so that self-antigens then could appear on MHC I and/or II molecules also on the surface of DC in the steady state *in vivo*. Al-

though the formation of MHC II/peptide complexes seems not to occur in immature DC [114]. The question remains however, why immature DC express MHC II molecules on their surface and what peptides are presented?

In conjunction with the low levels of surface MHC I and II expression, immature DC express only limited amounts of co-stimulatory molecules on their surface [115] and do not release pro-inflammatory cytokines [8, 116]. As indicated above, some B7 co-stimulation might be required to trigger CTLA-4 for induction [18] or maintenance of anergy [117].

26.5.3 Maturation Inhibitors

After isolation of DC from peripheral [118] or secondary lymphoid tissues [115] but also during the generation of murine BM-DC cultures [97, 119] DC mature "spontaneously", presumably due to the release of pro-inflammatory cytokines by different cell types of the disrupted tissue or macrophages developing in culture [120]. Thereby DC upregulate MHC and co-stimulatory molecules and acquire immunogenic properties. For this reason numerous methods have been developed to inhibit DC maturation and arrest the DC in a tolerogenic state (Table 26.2).

26.5.4 Maturation Resistance

Although the list of factors inhibiting DC maturation is constantly increasing, the stability of the immature phenotype has not been addressed by many studies. The induction of a stably immature phenotype, however, is necessary to maintain or improve the tolerogenic potential of the DC after injection. What this means is illustrated in Fig. 26.3, but has also been observed after injection of immature DC which then upregulated B7 molecules when detected in secondary lymphoid organs [121].

Maturation resistance has only been demonstrated by a few methods so far. The treatment of murine BM-DC cultures with only low doses of GM-CSF (5 U ml^{-1}) rendered the cells insensitive to maturation with TNF, LPS or anti-CD40 antibodies [122]. With these immature maturation-resistant DC, the mean heart allograft rejection time of 24 days that was reached with immature maturation-sensitive DC [121] could be extended to >120 days. Potent inhibitors of DC maturation in conjunction with maturation resistance found so far are: vitamin D3 analogs [123, 124]; the combined treatment of DC with IL-10 and TGF-β [125, 126]; and dexamethasone with human Mo-DC [127].

Tab. 26.2 Inhibitors of DC maturation.

Treatment or condition	***Maturation resistance***	***T-cell tolerance mechanism***	***References***
α-MSH			143
Aspirin		DTH inhibition	144, 145
Bordetella pertussis		IL-10⁺ reg T	85
CD47		n.d.	146–148
Diverse corticosteroids		anergy IL-10⁺ DC	149–155
Dexamethasone	yes		127
Cyclosporin A			151, 156–161
Early LPS		anergy	162
E-cadherin		n.d.	163
Fumaric acid esters		n.d.	164
Haptoglobin		n.d.	165
IL-10		anergy suppression	166–173
IL-10 plus TGF-β	yes		125, 126
Low GM-CSF, no IL-4	yes	anergy	122
Magnesium ions		n.d.	174
Malaria (*P. falciparum*)		dysregulation	175
Mycobacterium tuberculosis			176
Mycophenolate mofetil		n.d.	177
N-acetyl-L-cysteine		n.d.	178
Rapamycin			179
Sanglifehrin A			180
Soluble CD83		n.d.	181
Substance P		n.d.	182
TGF-β		deviation	120, 183–185
Trypanosoma cruzi		n.d.	186
UV-B light		anergy IL-12 p40 by DC	187–189
Vascular endothelial growth factor (VEGF)		n.d.	190
Viruses		diverse	191–198
Vitamin D3 analogs	yes	anergy	151, 199–202

n.d. = not determined

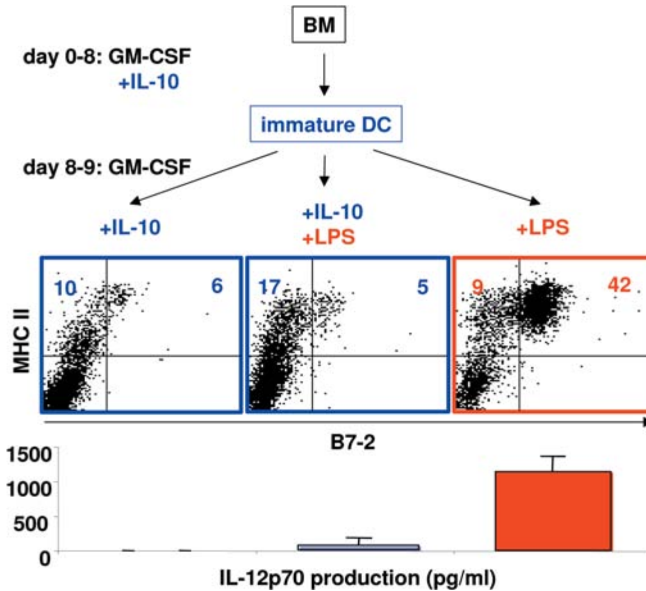

Fig. 26.3 Importance of maturation resistance by immature DC for their tolerogenicity. BM-DC were cultured for 8 days with GM-CSF (200 U ml^{-1}) plus IL-10 (10 ng ml^{-1}) to inhibit spontaneous maturation. Then the cells were washed and replated with GM-CSF and IL-10, or IL-10 plus LPS or LPS for another 24 h. FACS analysis was performed with the cells for surface MHC II (M5/114-PE) and B7-2 (FITC) and the IL-12p70 production measured by ELISA. The data show that in the absence of the inhibitory IL-10 signal, the immature DC rapidly mature on LPS.

26.6 Semi-mature DC

The term semi-mature was introduced to describe a stage of activation/maturation of DC that shows distinct characteristics of partial maturation. Semi-mature DC show an intermediate phenotype between immature DC, representing the resting tissue type, and completely mature DC, representing the migratory, T-cell priming type [8]. DC that have been suboptimally matured with TNF downregulated their endocytosis capacity and expressed high levels of DC maturation markers (CD25, CD205, 2A1), MHC II and co-stimulatory molecules, acquired lymph node homing potential (CCR7), but were unable to secrete cytokines or soluble mediators such as IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, NO and the chemokine MCP-1, in contrast to LPS or LPS plus anti-CD40 fully matured DC. Functionally, we found that such TNF/DC can induce peptide-specific tolerance in the Th1-mediated autoimmune model experimental autoimmune encephalomyelitis (EAE) [128]. The incomplete DC maturation we observed by TNF *in vitro*, has also been demonstrated by endogenous inflammatory mediators *in vivo*. Although $CD4^+$ T cells were activated, they could not induce any isotype switch in B cells [129]. The TNF/DC can be frozen and thawed for practical reasons. Intravenous injection is superior to the

intraperitoneal route and subcutaneous injection is not protective because the TNF/DC are co-localized and exposed to tissue injury signals counteracting their tolerogenicity (Fig. 26.4) [130].

More recent data indicate that semi-mature DC can be protective only in Th1 models such as EAE [128] or collagen-induced arthritis (CIA) [131] but not in Th2 models such as allergies [132], asthma (Erb/Lutz unpublished observations and [133]) or experimental autoimmune myocarditis (EAM) [134]. On the other hand LPS-matured BM-DC can ameliorate the Th2 model asthma [135] and TNF/DC can promote Th2 immunity in *L. major* infections in Th2-biased susceptible BALB/c mice (Fig. 26.4) (Wiethe/Gessner/Lutz manuscript in preparation). The latter point indicates that TNF/DC are also inducing Th2 immunity and might thereby immunedeviate the Th1 cells in the EAE model. This is even further supported by the fact that TNF/DC require co-activation of IL-4 and IL-13 secreting NKT cells through CD1d molecules on the DC surface for EAE protection [136]. Thus, TNF/DC induce a Th2/NKT2 shift which as a prerequisite to establish the protective Th2/Tr1 phenotype as observed upon repetitive administration of TNF/DC.

Other features of semi-mature DC generated by TNF treatment *in vitro* further underscore their relative rather than absolute tolerogenic potential. First, semi-mature DC are not terminally differentiated, since subsequent stimulation *in vitro* and

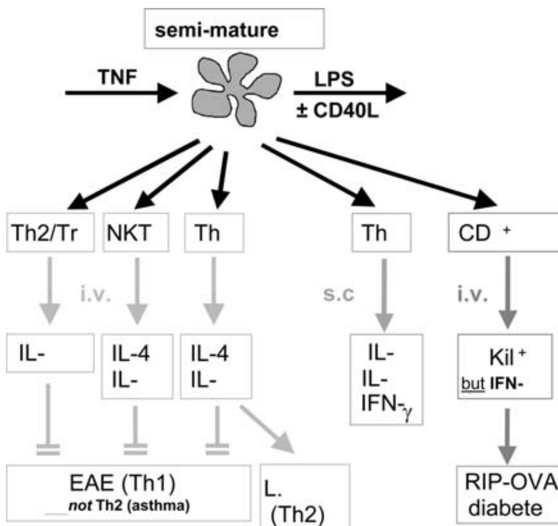

Fig. 26.4 Tolerogenic and immunogenic properties of *in vitro* generated semi-mature TNF/DC. Semi-mature DC are generated by stimulation with TNF and can further mature with LPS or LPS/anti-CD40 treatment. Repetitive i.v. injections of peptide-loaded DC will induce IL-10$^+$ IL-4$^+$ IL-13$^+$ CD4$^+$ T cells and IL-4$^+$ IL-13$^+$ NKT cells which in conjunction suppress Th1-mediated EAE but not Th2-mediated asthma. Injection of DC via the s.c. route abrogates protection by inducing an unpolarized Th0 response. Intravenous injection also promotes Th2 immunity in the *Leishmania* model and CD8$^+$ CTL activity in the RIP-OVA diabetes model despite application via the i.v. route. Thus semi-mature DC are only semi-tolerogenic depending on the type of T-cell response to be influenced. There however, they are highly effective and can suppress peptide-specifically.

in vivo by LPS or LPS plus anti-CD40 can convert them into fully mature DC, producing cytokines and abrogating their tolerogenic potential [130]. Second, by loading semi-mature DC simultaneously with peptides on MHC class I and II molecules we could show that these cells then tolerize the Th1 $CD4^+$ T-cell compartment for EAE induction, but immunize the $CD8^+$ T-cell compartment to induce CTL activity and induce diabetes in the transgenic RIP-OVA model. This demonstrates that $CD4^+$ and $CD8^+$ T cells have different co-stimulatory requirements for their activation (Fig. 26.4) [137].

26.6.1 Steady-state Migratory DC

Besides the *in vitro* generation of semi-mature DC the question remains as to whether such a differentiation stage of DC might exist *in vivo* and whether this correlate would then be tolerogenic as well. We outlined before that there is evidence that steady-state migratory DC in normal healthy mice represent the *in vivo* counterpart of *in vitro* generated semi-mature DC [8]. However our recent data indicate that semi-mature DC generated *in vitro* by TNF treatment induce TH2/NKT2 immune deviation, while the semi-mature steady state migratory DC *in vivo* might induce other mechanisms of CD4 T cell tolerance such as anergy and deletion (Kanazawa, Azukizawa, Lutz, in preparation).

In peripheral lymph nodes of healthy mice different lineage subsets of DC ($CD4^-8^-$, $CD4^+8^-$, $CD4^-8^+$, plasmacytoid) can be identified, but also different stages of maturation, i.e. immature $CD40^{low}$ and mature $CD40^{high}$ DC. The $CD40^{high}$ DC also express high levels of MHC II and B7 molecules and are migratory DC from the skin, thus representing Langerhans cells and dermal DC [73]. We extended these findings and found that the $CD40^{high}$ DC did not produce cytokines/chemokines (IL-6, IL-12p40, IL-10, MCP-1) apart from little TNF [138]. Because these $CD40^{high}$ DC very much resemble our *in vitro*-generated TNF/DC, we would argue that they are at a semi-mature stage.

The transport of tissue antigens or apoptotic material during the steady state has been demonstrated from the intestine [84], stomach [86], skin [83]. There is further evidence that such DC-mediated transport, processing and presentation of harmless antigens applied into the lung or expressed as a neo-self antigen will lead to T-cell tolerance by inducing $CD4^+$ Tr1 cells or $CD8^+$ deletion [52, 76, 87]. Such DC presenting self-antigens in the steady state on MHC I and II can appear mature as they express high levels of MHC I and II and co-stimulatory molecules but nevertheless act as if tolerogenic [74].

Thus, uptake of apoptotic material might partially activate the immature tissue resident DC for antigen processing, enhanced presentation (MHC II^{high}) and migration ($CCR7^{high}$) [139–141]. The resulting semi-mature, steady state migratory DC will present the transported self-antigens in a tolerogenic manner after arriving in the draining lymph node [8]. The mechanisms of T-cell tolerance might include induction of T-cell anergy/hyporesponsiveness [138], IL-10^+ reg T [52] and deletion [87].

26.7 Fully Mature DC

An interesting question is whether even fully mature DC might be able to induce certain forms of T-cell tolerance, despite the fact that their major task is the priming of adaptive immune responses.

Immunization studies showed that repetition of the priming process either with the help of adjuvants or by DC will boost the response, as can be measured by the percentages of specific T cells or antibody titers. Independent of the vaccination method, a maximum will be reached for each parameter. The intraclonal competition of specific T cells for antigen-presenting cells, including DC, which display the relevant peptide for restimulation, or simply the competition for space within the lymph node, might contribute to limit the amount of T-cell clonal expansion [29]. However, this is not a tolerogenic function of the antigen-priming DC.

Another aspect for the limitation of immune reactivity is the constitutive presence of the subset of $CD4^+$ $CD25^+$ T cells released from the thymus and detectable at 3–10% in the circulation and secondary lymphoid organs of mice and humans. The question remains as to which would be the type of DC able to activate $CD4^+$ $CD25^+$ T cells regulatory capacity *in vivo*. A proportion of $CD4^+CD25^+$ reg T permanently seems to express activation markers and divide in the steady state [75]. Adoptive transfer of OVA-specific $CD4^+$ $CD25^+$ TCR-transgenic DO11.10 T cells showed that only LPS-matured DC were able to expand the transferred reg T but not immature DC [54, 142]. These data indicate that $CD4^+$ $CD25^+$ reg T are co-induced with effector T cells to limit the immune response against foreign antigens. How the balance is regulated between priming and regulation remains elusive.

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Abbreviations

APC	antigen presenting cell	i.v.	intravenous
BM	bone marrow	pDC	plasmacytoid DC
DC	dendritic cells	reg T	regulatory T cells
LC	Langerhans cells	s.c.	subcutaneous

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27
Dendritic Cell Manipulation with Biological and Pharmacological Agents to Induce Regulatory T Cells

Luciano Adorini and Giuseppe Penna

27.1
Introduction

Dendritic cells (DCs), a highly specialized antigen-presenting cell (APC) system critical for the initiation of $CD4^+$ T-cell responses are present, in different stages of maturation, in the circulation as well as in lymphoid and nonlymphoid organs, where they exert a sentinel function. After antigen uptake, DCs migrate through the afferent lymph to T-dependent areas of secondary lymphoid organs where they can prime naïve T cells. During migration to lymphoid organs, DCs mature into potent APCs by increasing their immunostimulatory properties while decreasing antigen-capturing capacity [1].

It is now clear that DCs can be not only immunogenic but also tolerogenic, both intrathymically and in the periphery [2], and they can modulate T-cell development [3]. In particular, immature DCs have been found to have tolerogenic properties, and to induce T cells with suppressive activity [4]. However, the simplistic concept that immature DCs are intrinsically and uniquely able to induce suppressor T cells (Ts) has been dispelled by the observation that mature DCs can also be very efficient inducers of Ts cells [5], a property already noted for semi-mature DCs [6].

DCs are heterogeneous not only in terms of maturation state, but also of origin, morphology, phenotype and function [1, 7], and DC subsets have also been considered as specialized inducers of effector or suppressor T cells [8]. Two distinct DC subsets were originally defined in the human blood based on the expression of CD11c, and they have been subsequently characterized as belonging to the myeloid or lymphoid lineage. Although different denominations have been used, they can be defined as myeloid (M-DCs) and plasmacytoid (P-DCs) DCs [8, 9]. A cell population resembling human P-DCs has also been identified in the mouse [10]. M-DCs are characterized by a monocytic morphology; express myeloid markers like CD13 and CD33, the β2 integrin CD11c, the activatory receptor ILT1 and low levels of the IL-3 receptor α chain CD123. Conversely, P-DCs have a morphology resembling plasma cells, are devoid of myeloid markers, express high levels of CD4, CD62L and CD123. M-DCs produce high levels of IL-12, while P-DCs high

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levels of IFN-α [9], cytokines with clearly distinct effects on T-cell activation and differentiation.

Interest in the role of regulatory/suppressor T cells (Ts) cells has recently resurged and, among the various populations of Ts cells described, naturally-occurring thymic and peripheral $CD4^+$ T cells that co-express CD25 are currently the most actively investigated [11]. $CD4^+CD25^+$ Ts cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion [12]. They fail to proliferate and secrete cytokines in response to polyclonal or antigen-specific stimulation, and are not only anergic but also inhibit the activation of responsive T cells [11]. Although CD25, CD152, and glucocorticoid-induced TNF-related protein (GITR) are markers of $CD4^+CD25^+$ Ts cells, they are also expressed by activated T cells [11]. A more faithful marker distinguishing $CD4^+CD25^+$ Ts cells from recently activated $CD4^+$ T cells is Foxp3, a member forkhead family of transcription factors that is required for $CD25^+$Ts development and is sufficient for their suppressive function [13–15]. $Foxp3^+$ $CD4^+CD25^+$ Ts cells play an important role in preventing the induction of several autoimmune diseases, such as the autoimmune syndrome induced by day 3 thymectomy in genetically susceptible mice [12], inflammatory bowel disease [16], T1D in thymectomized rats [17] and in NOD mice [18, 19]. $CD25^+$Ts are reduced in NOD compared to other mouse strains, and this reduction could be a factor in their susceptibility to T1D [18, 20]. $CD25^+$Ts and effector T cells coexist within the pancreatic lesion before the onset of type 1 diabetes, and several factors, such as blockade of ICOS [21], can perturb this balance, precipitating autoimmunity. A defect in peripheral regulatory cells affecting both $CD25^+$Ts and NK cells has been described also in T1D patients [22], and autoreactive T cells in diabetics are skewed to a pro-inflammatory Th1 phenotype lacking the IL-10-secreting T cells found in nondiabetic, HLA-matched controls [23]. The clinical relevance of $CD4^+CD25^+$ Ts cells has also been shown in patients affected by rheumatoid arthritis [24] and multiple sclerosis [25].

Because DCs are pleiotropic modulators of T-cell activity, manipulation of DC function, to favor the induction of DCs with tolerogenic properties leading to the development of Ts cells, could be exploited to modulate immune responses. Considerable efforts are ongoing to translate this concept into clinical practice, also by rationalizing the tolerogenic effects exerted by immunosuppressive and immunomodulatory drugs currently used to control autoimmune diseases and graft rejection [26, 27].

27.2
Mechanisms Promoting Tolerogenic Dendritic Cells

Tolerogenic DCs are characterized by reduced expression of co-stimulatory molecules, in particular CD40, CD80, CD86. In addition, they show reduced IL-12 and increased IL-10 production, and often an early stage of maturation [2]. While these well-established phenotypic and functional properties of tolerogenic DCs can easily explain their propensity to induce regulatory rather than effector T cells,

several other mechanisms may play a role in favoring Ts cell induction by tolerogenic DCs.

27.2.1 Indoleamine 2,3-dioxygenase

One mechanism by which DCs can regulate T-cell responses is via expression of indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme of tryptophan catabolism. IDO-transgenic DCs decrease the concentration of tryptophan, increase the concentration of kynurenine, the main tryptophan metabolite, and suppress allogeneic T-cell proliferation *in vitro* due to T-cell death, because suppressive tryptophan catabolites exert a cytotoxic action preferentially on activated T cells [28]. Although the concept that cells expressing IDO can suppress T-cell responses and promote tolerance is a relatively new paradigm in immunology, accumulating evidence supports this possibility, including studies on maternal tolerance to the fetus, tumor resistance, chronic infections and autoimmune diseases [29]. In particular, IDO-expressing DCs contribute to the generation and maintenance of peripheral tolerance by depleting autorective T cells [29]. Interestingly, $CD4^+CD25^+$ Ts cells can condition DCs to express IDO functional activity and suppressive properties, a process that requires IFN-γ production by DCs [30].

A mechanistic link has also been established between the tolerance-inducing activity of CTLA-4-Ig and its capacity to induce IDO expression in DCs [31]. IDO-competent DC subsets acquire potent and dominant T-cell suppressive properties as a consequence of IDO upregulation, blocking the ability of T cells to respond to other stimulatory DCs in the same cultures [32]. Selective IDO upregulation in DCs does not inhibit T-cell activation, but prevents T-cell clonal expansion due to rapid death of activated T cells, suggesting that IDO-competent DCs provide a regulatory bridge, mediated by CTLA4-CD80/CD86 engagement, between regulatory and naïve responder T cells [32]. IDO expression is induced by CTLA-4-Ig in specific DC subsets, notably in $CD8\alpha^+$ and plasmacytoid DCs, and provides a potential mechanistic explanation for their T-cell regulatory properties [33]. Mouse plasmacytoid DCs can also initiate the immunosuppressive pathway of tryptophan catabolism, via type I IFN receptor signaling, in response to CD200 receptor engagement [34].

Human DCs that express IDO also inhibit T-cell proliferation *in vitro* [35]. Interestingly, IDO-mediated suppressor activity is present in fully mature as well as immature human $CD123^+$ plasmacytoid DCs. IDO^+ DCs can be readily detected *in vivo*, suggesting that these cells may represent a regulatory subset of APCs in man [36]. Thus, inducible IDO expression appears to play an important role in making DC tolerogenic, and its modulation has potential therapeutic applications.

27.2.2 Immunoglobulin-like Transcripts

Immunoglobulin-like transcripts (ILTs) are receptors structurally and functionally related to killer cell inhibitory receptors (KIR) [37] that have been shown to be in-

volved in immunoregulation [38]. ILT family members can be subdivided into two main types. One, comprising ILT1, ILT7, ILT8, and leukocyte Ig-like receptor 6, is characterized by a short cytoplasmic tail delivering an activating signal through the immunoreceptor tyrosine-based activatory motif (ITAM) of the associated common γ chain of the Fc receptor. Members of the second type, including ILT2, ILT3, ILT4, and ILT5, contain a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) transducing a negative signal [39]. When inhibitory ILTs are activated, their ITIM domains become phosphorylated, and recruit $p56^{lck}$ and SH2-containing protein-tyrosine-phosphatase 1 (SHP-1), leading to downstream events and gene modulation [40]. The high homology between ILTs and KIRs suggests that ILTs can also interact with class I MHC molecules, but this has been so far confirmed only for ILT2 and ILT4 [41, 42].

Most cell types involved in innate or acquired immune responses, including myeloid, lymphoid and dendritic cells, express at least one member of the ILT family, which may play an important role in immunoregulation [40]. For example, the inhibitory receptor ILT3 has been shown to negatively regulate activation of antigen-presenting cells [43]. A connection between ILTs and tolerance induction has been established by the observation that $CD8^+CD28^-$ suppressor T cells upregulate ILT3 and ILT4 expression on DCs, rendering them tolerogenic [44]. Such tolerogenic DCs have been reported to anergize alloreactive $CD4^+CD45RO^+CD25^+$ T cells converting them into regulatory T cells which, in turn, continue the cascade of suppression by tolerizing other DCs [45]. Alloantigen specific $CD8^+CD28^-$ $Foxp3^+$ T suppressor cells have also been shown to induce $ILT3^+$ $ILT4^+$ tolerogenic endothelial cells, inhibiting alloreactivity [46]. Consistent with these results, rat $CD8^+Foxp3^+$ Ts cells have been shown to induce PIR-B, an hortolog of inhibitory ILTs [47], in DCs and heart endothelial cells, and to mediate tolerance to allogeneic heart transplants [48].

27.3 Induction of Tolerogenic Dendritic Cells

Dendritic cells induce and regulate T-cell responses, and tolerogenic DCs can promote the development of regulatory T cells with suppressive activity. Thus, the possibility to manipulate DCs using different pharmacological or biological agents, enabling them to exert tolerogenic activities, could be exploited to better control a variety of chronic inflammatory conditions, from autoimmune diseases to allograft rejection. Both biological and pharmacological agents have been shown capable of inducing tolerogenic DCs [26, 27, 49]. Notably, several *in vitro* studies have demonstrated that human regulatory T cells can be induced by DCs manipulated to acquire and/or enhance tolerogenic properties, and *in vivo* data are also accumulating.

27.3.1 Biological Agents Promoting Tolerogenic Dendritic Cells

Cytokines represent the best known class of biological agents currently used to favor the induction of tolerogenic DCs. In particular, DCs differentiated in the presence of IL-10, TGF-β, TNF-α, or G-CSF can acquire phenotypic and functional properties characteristic of tolerogenic DCs.

27.3.1.1 IL-10

Although several biological agents can favor, directly or indirectly, induction of regulatory T cells, IL-10 is probably the best example of an immunomodulatory protein that promotes regulatory T-cell induction, at least in part, by targeting DCs.

APCs and T cells are primary targets of IL-10, a potent suppressor of several effector functions of macrophages, dendritic cells, T cells and NK cells. In addition, IL-10 contributes to regulate proliferation and differentiation of B cells, mast cells, and thymocytes [50]. An important property of IL-10, from an immunotherapeutic perspective, is its capacity to inhibit Th1 cells. The inhibition of the Th1 cell pathway by IL-10 is mediated by several mechanisms, including inhibition of IL-12 production by APCs, and blocking of IFN-γ synthesis by differentiated Th1 cells [50]. However, IL-10 targets more efficiently naïve rather than activated or memory T cells, possibly due to the downregulation of IL-10R α chain. In addition, IL-10 strongly inhibits production of pro-inflammatory monokines as IL-1, IL-6, IL-8, TNF-α and GM-CSF, as well as of reactive oxygen and nitrogen species following activation of human or mouse macrophages. The intrinsically strong anti-inflammatory properties of IL-10 are further enhanced by its capacity to induce regulatory T cells. In particular, the presence of IL-10 during differentiation of $CD4^+$ T cells results in the development of a defined subset of regulatory T cells (Tr1 cells) characterized by low proliferation, absence of IL-2 production, and a specific cytokine profile characterized by IL-10 and IFN-γ but no IL-4 nor IL-5 production [51].

It is possible that these inhibitory functions of IL-10 and related cytokines can find therapeutic applications, and their activity in modulating APC functions and in promoting development of regulatory T cells suggests a possible use in the treatment of autoimmune diseases [52]. Supporting this hypothesis, severe colitis was abrogated in a model of inflammatory bowel disease by systemic administration of IL-10 but, interestingly, not of IL-4. In addition, IL-10 treatment can ameliorate mouse lupus via inhibition of pathogenic Th1 cytokines [50]. IL-10 treatment is currently being tested in multiple inflammatory conditions, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, allograft rejection and chronic hepatitis C [53]. The results from clinical trials are heterogeneous but, so far, inferior to the expectations [53].

27.3.1.2 TGF-β

TGF-β, a member of a large family of evolutionary conserved proteins known for their pleiotropic activities, can promote or inhibit cell growth and function. TGF-β1 is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells. It can modulate expression of adhesion molecules, provide a chemotactic gradient for leukocytes and other cells participating in an inflammatory response, and inhibit them once they have become activated [54].

The important role of TGF-β in autoimmune diseases is shown by the massive autoimmune inflammation affecting multiple organs in mice deficient in TGF-β or with induced disruption of the TGF-β type II receptor, as well as, for example, by the inhibition of EAE following TGF-β administration, and by enhancement of EAE upon its neutralization [54]. In addition, TGF-β is considered a major mediator in oral tolerance [55]. Although the disease-limiting properties of TGF-β in autoimmune diseases seem attractive, disruption of the balance between its opposing activities can contribute to aberrant development, malignancy, or pathogenic immune and inflammatory responses characterized by widespread tissue fibrosis and deposition of extracellular matrix, as shown by the reversible decline in the glomerular filtration rate observed in an open-label trial in patients with secondary progressive MS [56].

Several studies have shown that TGF-β1 inhibits *in vitro* activation and maturation of DCs, preventing the upregulation of critical T-cell co-stimulatory molecules on DC surface, inhibiting IL-12 production, and reducing their antigen-presenting capacity [57–59]. Thus, in addition to direct inhibitory effects of TGF-β1 on effector T lymphocytes [60], and to induction of the Ts transcription factor foxp3 [61], its inhibitory effects at the DC level may critically contribute to the immunosuppressive effects. In contrast to these negative regulatory effects of TGF-β1 on DC function and maturation, certain subpopulations of immature DCs in non lymphoid tissues are positively regulated by TGF-β1 signaling. In particular, epithelial-associated DC populations seem to critically require TGF-β1 stimulation for development and function, as shown for the development of epithelial Langerhans cells *in vitro* and *in vivo*, in which TGF-β1 seems to enhance also antigen processing and co-stimulatory functions [62].

27.3.1.3 TNF-α

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine with interesting immunoregulatory properties [63], that can modulate DC development, phenotype and function [6]. Maturation by TNF-α induce high levels of MHC class II and co-stimulatory molecules on DCs, but they remain weak producers of pro-inflammatory cytokines, in particular IL-12 [6]. These incompletely matured DCs (semi-mature DCs) induce peptide-specific IL-10-producing T cells *in vivo* and prevent EAE [64]. DCs with a similar phenotype were previously found to inhibit, when injected 1 week before transplantation, haplotype-specific cardiac allograft rejection, with a marked increase in median graft survival time from 8 to >100 days [65]. Injection of semi-mature DCs could also protect mice from GVHD and induce the expan-

sion of IL-10-producing $CD4^+CD25^+$ Ts cells [66], suggesting that semi-mature DCs may be beneficial in the treatment of several immune-mediated diseases.

27.3.1.4 G-CSF

Granulocyte colony-stimulating factor (G-CSF), the key hematopoietic growth factor of the myeloid lineage, has been recently found to possess marked immunoregulatory properties [67, 68].

$CD4^+$ T cells exposed *in vivo* to G-CSF acquire Tr1-type properties, once triggered *in vitro* through the T-cell receptor, including IL-10-dominated cytokine production profile, intrinsically low proliferation, and contact-independent suppression of antigen-driven proliferation [69]. The immunomodulatory effects of G-CSF might be mediated by DCs expressing high levels of co-stimulatory molecules and HLA-DR, but decreased IL-12p70 secretion and poor allostimulatory capacity [70], reminiscent of semi-mature DCs [6]. The ability of G-CSF to promote key tolerogenic interactions between DCs and regulatory T-cells has been recently demonstrated by the enhanced recruitment of TGF-β1-expressing $CD4^+CD25^+$ Ts cells after adoptive transfer of DCs isolated from G-CSF- compared to vehicle-treated mice into naïve NOD recipients [71].

27.3.2 Pharmacological Agents Promoting Tolerogenic Dendritic Cells

A variety of immunosuppressive agents are currently used to inhibit transplantation rejection and to treat autoimmune diseases (Table 27.1). Some of these pharmacological agents have been instrumental in the control of allograft rejection, giving a decisive impulse to clinical transplantation in the late 1970s. Interestingly, the mechanism of action of major immunosuppressive drugs, like the calcineurin inhibitors cyclosporine A and tacrolimus, has been only understood after almost 20 years of clinical use [72]. Thus, it is perhaps not surprising that a novel mechanism of action shared by many immunosuppressive and anti-inflammatory agents, based on the induction of DCs with tolerogenic properties, has only recently emerged [26, 27, 49, 73].

Indeed, several immunosuppressive agents currently used to treat allograft rejection and autoimmune diseases have been shown to induce DCs with tolerogenic phenotype and function (Table 27.1). Notable examples are glucocorticoids [74–77], mycophenolate mofetil (MMF) [78, 79], and sirolimus [80, 81]. These agents impair DC maturation and inhibit upregulation of co-stimulatory molecules, secretion of pro-inflammatory cytokines, in particular IL-12, and allostimulatory capacity. Sirolimus appears to be a very interesting agent, because it induces tolerogenic DCs [81], and sirolimus-treated alloantigen-pulsed DCs infused 1 week before transplantation inhibit antigen-specific T-cell responsiveness and prolong graft survival [82].

Conversely, controversial effects of calcineurin inhibitors, like cyclosporine A and tacrolimus, have been reported on DC maturation, although these drugs have

a clear inhibitory effect on DC, decreasing their cytokine production and allostimulatory capacity [77, 83, 84]. Other immunosuppressive agents, like desoxyspergualin, also inhibit the allostimulatory capacity of DCs, impairing their maturation and IL-12 production as well [85–87]. Similar effects are exerted on DCs by anti-inflammatory agents, such as acetylsalicylic acid [88, 89], butyric acid [90] and *N*-acetyl-L-cysteine [91].

AGENT	EFFECTS ON DENDRITIC CELL						
BIOLOGICAL	Differentiation	Maturation	Costimulatory molecules	IL-12 Production	IL-10 Production	Allostimulatory capacity	NF-kB Activation
IL-10	↓	↓	↓	↓		↓	
TGF-β	↓	↓	↓			↓	
TNF-α		↓	↑	↓		↓	
G-CSF		↓	↑	↓		↓	
PHARMACOLOGICAL							
Acetylsalicylic acid		↓	↓	↓	↔	↓	↓
Butyric acid	↓	↓	↓				
Calcineurin inhibitors	↔	↓	↓	↓	↔	↓	↓
Deoxyspergualin		↓	↓	↓		↓	↓
Glucocorticoids	↓	↓	↓	↓	↔	↓	↓
N-acetyl-L-cysteine		↓	↓	↓		↓	↓
Mycophenolate mofetil	↓	↓	↓	↓		↓	
Sirolimus	↓	↓	↓	↓		↓	
Vitamin D receptor agonists	↓	↓	↓	↓	↑	↓	↓

Tab. 27.1 Effects of pharmacological and biological agents with anti-inflammatory and immunosuppressive properties on dendritic cells. Compiled from references quoted in the text. Downwards arrows indicate inhibition, upwards arrows stimulation, and horizontal arrows no effect. Blanks indicate information not available.

Although the pro-tolerogenic effects of several pharmacological agents on DCs are well established, little is known about their capacity to induce regulatory T cells promoting transplantation tolerance. MMF was able, as a monotherapy, to induce some limited levels of transplantation tolerance even if no induction of tolerogenic DCs was observed *in vivo* [92]. Conversely, calcineurin inhibitors have been reported to prevent transplantation tolerance induced by co-stimulation blockers, although the issue is still unresolved [93], but successful establishment of alloantigen-specific hyporesponsiveness by NF-_B inhibitor-treated DCs was not inhibited by concomitant calcineurin inhibition [94]. In addition, the sirolimus derivative everolimus did not hamper *in vitro* the suppressive activity of $CD4^+CD25^+$ Ts cells, suggesting that these cells may still exert suppressive activity in transplant recipients treated with drugs interfering with IL-2 signaling [95].

Finally, as discussed in greater detail below, the activated form of vitamin D, $1,25(OH)_2D_3$, and its analogues have also been found to inhibit DC maturation, leading to reduced expression of co-stimulatory molecules and IL-12 production. These tolerogenic DCs show decreased capacity to stimulate alloreactive T cells, and promote the differentiation of $CD4^+CD25^+$ Ts cells.

27.4 Induction of Tolerogenic Dendritic Cells by VDR Agonists

The activated form of vitamin D, $1,25(OH)_2D_3$, is a secosteroid hormone that has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronounced immunoregulatory properties [96–100]. The biological effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors functioning as a agonist-activated transcription factor that binds to specific DNA sequence elements, vitamin D responsive elements, in vitamin D responsive genes and ultimately influences their rate of RNA polymerase II-mediated transcription [101].

APCs, and notably DCs, express the VDR and are key targets of VDR agonists, both *in vitro* and *in vivo*. A number of studies, summarized in Table 27.2, has clearly demonstrated that $1,25(OH)_2D_3$ and its analogues markedly modulate DC phenotype and function [102–107]. These studies, performed either on monocyte-derived DCs from human peripheral blood or on bone-marrow derived mouse DCs, have consistently shown that *in vitro* treatment of DCs with $1,25(OH)_2D_3$ and its analogues leads to downregulated expression of the co-stimulatory molecules CD40, CD80, CD86, and to decreased IL-12 and enhanced IL-10 production, resulting in decreased T-cell activation (Table 27.2). The block of maturation, coupled with abrogation of IL-12 and strongly enhanced production of IL-10, highlight the important functional effects of $1,25(OH)_2D_3$ and its analogues on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties. The combination of these effects can explain the capacity of VDR agonists to induce DCs with tolerogenic properties that favor suppressor T-cell enhancement. DCs

are able to synthesize $1,25(OH)_2D_3$ *in vitro* as a consequence of increased 1α-hydroxylase expression [108], and this could also contribute to promote regulatory T-cell induction. It is also possible that $1,25(OH)_2D_3$ may contribute to the physiological control of immune responses, and possibly be also involved in maintaining tolerance to self antigens, as suggested by the enlarged lymph nodes containing a higher frequency of mature DCs in VDR-deficient mice [109].

Tab. 27.2 Phenotypic and functional modifications induced by VDR ligands in human myeloid dendritic cells.

Phenotype	***Effect***
Maturation marker expression	
CD83	decreased
DC-LAMP	decreased
Antigen uptake	
Mannose receptor expression	increased
Co-stimulatory molecule expression	
CD40	decreased
CD80	decreased
CD86	decreased
Inhibitory molecule expression	
ILT3	increased
ILT4	unmodified
B7-H1	unmodified
Chemokine receptor expression	
CCR7	decreased
Function	***Effect***
Cytokine production	
IL-10	increased
IL-12	decreased
Chemokine production	
CCL2	increased
CCL17	decreased
CCL18	increased
CCL20	decreased
CCL22	increased
Apoptosis	
Maturation-induced	increased
T-cell activation	
Response to alloantigens	decreased

Compiled from refs. [102, 134] and from the author's unpublished data.

27.4.1 Tolerogenic Dendritic Cells Induced by VDR Agonists lead to enhancement of regulatory T cells

The prevention of DC differentiation and maturation as well as the modulation of their activation and survival leading to DCs with tolerogenic phenotype and function (Table 27.2) play an important role in the immunoregulatory activity of VDR agonists, and appear to be critical for the capacity of this hormones to induce $CD4^+CD25^+$ Ts cells that are able to control autoimmune responses and allograft rejection (Table 27.3).

VDR agonists enhance $CD4^+CD25^+$ Ts cells and promote tolerance induction in transplantation and autoimmune disease models. A short treatment with $1,25(OH)_2D_3$ and mycophenolate mofetil, a selective inhibitor of T and B cell proliferation that also modulates APCs, induces tolerance to islet allografts associated with an increased frequency of $CD4^+CD25^+$ Ts cells able to adoptively transfer transplantation tolerance [92]. The induction of tolerogenic DCs could indeed represent a therapeutic strategy promoting tolerance to allografts [99] and the observation that immature myeloid DCs can induce T-cell tolerance to specific antigens in human volunteers represents an important proof of concept for this approach [110].

$CD4^+CD25^+$ Ts cells able to inhibit the T-cell response to a pancreatic autoantigen and to significantly delay disease transfer by pathogenic $CD4^+CD25^-$ T cells are also induced by treatment of adult nonobese diabetic (NOD) mice with the VDR agonist BXL-219 [111]. This treatment arrests insulitis, blocks the progression of Th1 cell infiltration into the pancreatic islets, and inhibits type 1 diabetes development at nonhypercalcemic doses [111]. Although the type 1 diabetes and islet transplantation models are quite different, in both cases administration of VDR

Tab. 27.3 VDR agonists foster the induction of regulatory T cells.

In dendritic cells
Inhibit IL-12
Enhance IL-10
Down-regulate CD40, CD80, CD86
Block maturation
Upregulate ILT3 expression
Leading to T cells characterized by
Reduced Th1 development
Hyporesponsiveness to auto and alloantigens
Increased CTLA-4 expression
Decreased CD40L expression
Enhanced $CD4^+CD25^+$ suppressor T cells

Compiled from references [102–107, 115] and from the author's unpublished results.

agonists doubles the number of $CD4^+CD25^+$ Ts cells, in the spleen and pancreatic lymph nodes, respectively.

However, tolerogenic DCs may not always be necessarily involved in the generation of Ts cells by VDR agonists. A combination of $1,25(OH)_2D_3$ and dexamethasone has been shown to induce human and mouse naïve $CD4^+$ T cells to differentiate *in vitro* into Ts cells, even in the absence of APCs [112]. These Ts cells produced IL-10, but no IL-5 nor IFN-γ, thus distinguishing them from the previously described Tr1 cells [51]. Upon transfer, the IL-10-producing Ts cells could prevent central nervous system inflammation, indicating their capacity to exert a suppressive function *in vivo* [112]. Thus, although DCs appear to be primary targets for the immunomodulatory activities of VDR agonists, they can also act directly on T cells, as expected by VDR expression in both cell types and by the presence of common targets in their signal transduction pathways, such as the nuclear factor NF-κB that is downregulated in APCs and in T cells.

27.4.2
Upregulation of Inibitory Receptor Expression in Dendritic Cells by VDR agonists

To further characterize mechanisms accounting for the induction of DCs with tolerogenic properties by VDR agonists, we have examined the expression of immunoglobulin-like transcripts (ILT), receptors structurally and functionally related to killer cell inhibitory receptors (KIR) [43], by $1,25(OH)_2D_3$-treated DCs. We have found that incubation of monocyte-derived human DCs, either immature or during maturation, with $1,25(OH)_2D_3$ leads to a selective upregulation of ILT3 [27]. Analysis of DC subsets revealed a higher ILT3 expression on P-DCs compared to M-DCs [113, 114]. CD40 ligation reduced ILT3 expression on M-DCs but had little effect on P-DCs [115]. Maintaining high ILT3 expression on P-DCs matured via CD40 ligation is of interest, because this cell population has been shown to induce $CD8^+$ regulatory T cells able to suppress the proliferation of naïve $CD8^+$ cells through an IL-10-dependent pathway [116]. While incubation with $1,25(OH)_2D_3$ did not affect the already high ILT3 expression by P-DCs, it increased its expression on M-DCs considerably [115]. The downregulation of ILT3 on M-DCs by T cell-dependent signals, and the upregulation of this inhibitory receptor by $1,25(OH)_2D_3$ in DCs suggests a novel mechanism for the immunomodulatory properties of this hormone that could play a role in the control of T-cell responses.

As tolerogenic DCs induced by different pharmacological agents share several properties (Table 27.1), we analyzed upregulation of ILT3 expression in immature and mature DCs by selected immunomodulatory agents. $1,25(OH)_2D_3$ markedly upregulates ILT3 expression on both immature and mature DCs, whereas IL-10 has a much less pronounced effect, and dexamethasone no observable activity. In the same experiment, all the three agents inhibited DC maturation, as shown by decreased CD83 expression [27]. An *in vivo* correlate could be established by the marked upregulation of ILT3 expression in DCs of psoriatic lesions treated with the VDR agonist calcipotriol, whereas no ILT3 expression was induced by topical treatment of psoriatic plaques with the glucocorticoid mometazone [135]. These re-

sults indicate that drug-induced ILT3 upregulation is not a general feature of tolerogenic DCs, as proposed by a recent study [117], and are consistent with the view that VDR agonists and glucocorticoids modulate DCs using distinctive pathways [118]. Although ILT3 expression by DCs is required for induction of regulatory T cells, DC pretreatment with $1{,}25(OH)_2D_3$ leads to induction of $CD4^+Foxp3^+$ cells with suppressive activity irrespective of the presence of neutralizing anti-ILT3 mAb, indicating that ILT3 expression is dispensable for the capacity of $1{,}25(OH)_2D_3$-treated DCs to induce regulatory T cells (ref. Penna, G., A. Roncari, S. Amuchastegui, K. C. Daniel, E. Berti, M. Colonna, and L. Adorini. 2005. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of $CD4^+Foxp3^+$ regulatory T cells by 1,25-dihydroxyvitamin D3. *Blood 106:* 3490–3497).

27.4.3
Modulation of Chemokine Production by VDR Agonists can affect Recruitment of Effector T cells and $CD4^+CD25^+$ Ts cells to Inflammatory Sites

In both islet transplantation and type 1 diabetes models, treatment with VDR agonists has a profound effect on the migration of effector T cells, preventing their entry into the pancreatic islets [92, 111]. The VDR agonist BXL-219 significantly downregulates *in vitro* and *in vivo* pro-inflammatory chemokine production by islet cells, inhibiting T-cell recruitment into the pancreatic islets and T1D development [119]. The inhibition of CXCL10 is particularly relevant, consistent with the decreased recruitment of Th1 cells into sites of inflammation by treatment with an anti-CXCR3 antibody [120], and with the substantial delay of T1D development observed in CXCR3-deficient mice [121]. The inhibition of islet chemokine production by BXL-219 treatment *in vivo* is associated with upregulation of $I\kappa B\alpha$ transcription, an inhibitor of nuclear factor κB (NF-κB), and with arrest of NF-κBp65 nuclear translocation [119], highlighting a novel mechanism of action exerted by VDR agonists potentially relevant for the treatment of T1D and other autoimmune diseases. These observations expand the known mechanisms of action exerted by vitamin D analogs in the treatment of T1D and other autoimmune diseases, that include arrest of DC maturation, inhibition of Th1 cell responsiveness, and enhancement of regulatory T cells [97, 98, 100]. In addition to modulating chemokine production in target tissues such as pancreatic islets, it is also possible that VDR agonists can affect the migration of $CD25^+$Ts cells by regulating their chemokine receptor expression, a hypothesis that we are currently testing.

Both human [122] and mouse (N. Giarratana et al., submitted for publication) $CD4^+CD25^+$ Ts cells express CCR4, and selectively migrate in response to CCR4 agonists like CCL22. An interesting confirmation to this finding is provided by the observation that human ovarian tumors produce CCL22, the cognate ligand of the CCR4 receptor, promoting the recruitment of $CCR4^+$ $CD4^+CD25^+$ Ts cells that act as a tumor-protective mechanism [123].

We have found that, in contrast to the high production by circulating human myeloid DCs (M-DCs), the CCR4 agonists CCL17 and CCL22 are poorly produced by plasmacytoid DCs (P-DCs) [124]. It is noteworthy that blood-borne M-DCs, in

contrast to P-DCs, constitutively produce CCL17 and CCL22 *ex vivo* [124]. This selective constitutive production of CCR4 agonists by immature M-DCs could lead to the preferential attraction of $CD4^{+}CD25^{+}$ Ts cells, a mechanism expected to favor tolerance induction. This has been observed in ovarian carcinoma patients, in which $Foxp3^{+}CCR4^{+}CD25^{+}$Ts cells are selectively recruited by tumor-produced CCL22, and suppress antitumor responses leading to reduced patient survival [123]. Intriguingly, the production of CCL22 is markedly enhanced by $1,25(OH)_2D_3$ in blood M-DCs but not P-DCs (Penna et al., manuscript in preparation). Besides maintaining peripheral immunological tolerance in homeostatic conditions, $CD4^{+}CD25^{+}$ Ts cells could turn-off and limit ongoing inflammatory responses. Inflammatory signals strongly induce maturation and influx of both M-DCs and P-DCs to secondary lymphoid tissues [8], and maturation of M-DCs and P-DCs enhances their production of several pro-inflammatory chemokines that can potentially attract different T-cell subsets. Interestingly, maturing P-DCs, similarly to activated B cells, produce large quantities of the CCR5 agonist CCL4 [124]. Thus, in analogy with the proposed role for CCL4 in $CD4^{+}CD25^{+}$ Ts cells attraction by activated B cells, mature P-DCs could recruit these cells to limit ongoing inflammatory responses.

27.5
Common Features of Agents Leading to Induction of Tolerogenic DCs

As summarized in Table 27.1, common features shared by biological and pharmacological agents favoring the induction of tolerogenic DCs are their capacity to inhibit differentiation, maturation, co-stimulatory molecule expression, and IL-12 production, leading to decreased allostimulatory capacity.

Co-stimulatory molecule expression is almost invariably reduced in tolerogenic DCs, with the exception of exposure to agents inducing semi-mature DCs [6]. In any case, all the tolerogenic agents tested inhibit DC maturation and reduce their capacity to stimulate alloreactive T cells in a mixed leukocyte reaction assay. Another common feature of DC-targeting drugs is the inhibition of IL-12, a cytokine critically involved in the development of Th1-dependent diseases [125]. In contrast, only $1,25(OH)_2D_3$ and its analogues, among the agents tested, are able to enhance the secretion by DCs of IL-10, a cytokine favoring the induction of regulatory T cells (Table 27.1).

Several of these effects could be mediated by NF-κB, a signal transduction pathway crucially involved in the inflammatory response [126]. The NF-κB family member RelB is required for myeloid DC differentiation, and antigen-pulsed DCs in which RelB function is inhibited can induce regulatory $CD4^{+}$ T cells able to transfer tolerance to primed recipients in an IL-10-dependent fashion [127]. Our data showing upregulation of transcripts encoding IκBα and inhibition of RelA nuclear translocation by BXL-219 in pancreatic islet cells [119] demonstrate a novel mechanism of action in the targeting NF-κB by VDR ligands, in addition to inhibition of NF-κB1 and c-Rel [128], as well as RelB [129] expression. Interestingly, this mechanism of action has been previously demonstrated for glucorticoids, anti-inflammatory drugs that bind to a nuclear receptor in the same superfamily as the

VDR, by showing that dexamethasone upregulates the transcription of *Nfkbia*, which results in increased rate of IκBα synthesis and in reduced NF-κB translocation to the nucleus [130, 131]. The upregulation of transcripts encoding IκBα and the inhibition of RelA translocation to the nucleus by BXL-219 prevent activation of NF-κB, a transcription factor that also regulates chemokine production by pancreatic β cells [132]. The promoter of the *Nfkbia* gene encoding IκBα contains, as the *Relb* gene [129], several vitamin D responsive elements, some of which are highly conserved between human and mouse homologs, suggesting a direct transcriptional regulation of IκBα by BXL-219 [119]. The direct targeting of NF-κB components by VDR agonists could thus contribute to explain their inhibition of pro-inflammatory cytokine and chemokine production by DCs, as well as the inhibition of DC maturation, and could open new avenues in the use of VDR agonists as anti-inflammatory agents.

27.6 Conclusions

Several immunomodulatory agents, and in particular immunosuppressive and anti-inflammatory drugs, share the capacity to target DCs, rendering them tolerogenic and fostering the induction of regulatory rather than effector T cells. Multiple mechanisms contribute to induction of DC tolerogenicity, from downregulation of co-stimulatory molecules, both membrane-bound as CD40, CD80, CD86 and secreted as IL-12, to upregulation of inhibitory molecules like IDO and ILT3, to modulation of chemokine secretion, enhancing the production of chemokines able to recruit regulatory/suppressor T cells, and inhibiting chemokine production by the target organ in inflammatory conditions.

In principle, these mechanisms favoring DC tolerogenicity could be exploited in two ways. The first could rely on the *in vitro* manipulation of DCs to promote tolerogenic properties, followed by reinfusion into the patient. However, this, as any cell-based therapy, poses tremendous hurdles for clinical applicability. The standardization of effective and reproducible protocols would be very difficult, but even more problematic will be to ensure the capacity of the reinfused DCs to maintain a tolerogenic function *in vivo*, under inflammatory conditions. In contrast to therapies based on *ex-vivo* manipulation of DCs, treatments with immunosuppressive and anti-inflammatory drugs able to promote tolerogenic DCs have been in clinical use for decades to control allograft rejection and autoimmune diseases. Administration of these agents can directly target both DCs and T cells, leading to the inhibition of pathogenic effector T cells and enhancing the frequency of T cells with suppressive properties, effects that appear to be largely mediated via induction of tolerogenic DCs. Thus, direct treatment of patients with DC-tolerizing agents appears to represent a preferable therapeutic option. In addition to low molecular weight drugs, biological agents, in particular cytokines, could be directly administered *in vivo* to promote induction of tolerogenic DCs. However, issues related to immunogenicity, short and long-term adverse events, and high cost are likely to place important limitations on their clinical applicability.

Our own work has explored the immunoregulatory activities of VDR agonists, secosteroid hormones able to induce tolerogenic DCs and regulatory T cells. VDR agonists have been proven effective and safe drugs in a variety of autoimmune disease [96, 98] and graft rejection [99, 133] models, highlighting their potential applicability in chronic inflammatory conditions sustained by autoreactive or alloreactive immune responses. In addition to the topical treatment of psoriasis, a Th1-mediated autoimmune disease of the skin where VDR agonists are the most used topical drugs, these agents might eventually find a broader application in the treatment of inflammatory conditions, where their modulatory effects on DCs enhancing T cells with regulatory functions could turn out to be highly beneficial.

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28
Surface Molecules Involved in the Induction of Tolerance by Dendritic Cells

Laura C. Bonifaz

28.1
Introduction

To prevent autoimmunity and collateral damage during ongoing immune responses, T lymphocytes, as components of the adaptive immune system, need to remain tolerant to self- and innocuous environmental antigens (Ag). As a specific process, Ag are needed for the induction of tolerance. Although for central tolerance, the transcription factor autoimmune regulator protein (AIRE) [1], and possibly others, permits the expression of genes that encode tissue-specific Ag by thymic medullary epithelial cells, it is unlikely that all self-Ag and innocuous environmental Ag can be present in the thymus at any given time. Therefore, additional mechanisms of tolerance in peripheral lymphoid tissue are important to avoid undesirable immune responses.

The first step in the induction of tolerance to Ag that are not expressed by antigen-presenting cells is the sampling of these exogenous proteins by endocytic receptors, which is followed by processing and presentation to antigen-specific T cells of peptides, bound to MHC molecules. Dendritic cells (DC), as efficient antigen-capturing and processing cells, are appropriate candidates to participate in the induction of the tolerance process. Several questions need to be answered in order to understand the circumstances under which DC, known for their specialized immunizing properties, also participate in the opposing processes of tolerance.

Tolerance, by definition, is a physiological state of unresponsiveness. To ensure this state, three major mechanisms are currently accepted, which are thought to involve: (a) clonal deletion, (b) clonal anergy, and (c) different types of T regulatory cells such as the natural $CD4^+$ $CD25^+$ variety.

Clonal deletion appears to be the main mechanism for central tolerance in the thymus [2]. However, it has also been shown to operate in the periphery. Anergy has been described mainly in the periphery, and in some experimental models is still regarded as controversial [3]. $CD4^+CD25^+$ T cells are generated during development in the thymus, but they also can be expanded and possibly induced *de novo* by DC in the periphery [4].

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28.2 Dendritic Cells and Central Tolerance

In addition to medullary epithelial cells, bone-marrow derived DC play an important role in the induction of central tolerance. As mentioned, the main mechanism of tolerance in the thymus is activation-induced cell death, which leads to deletion of those T cells that recognize self-peptides bound to MHC class I and class II molecules on the surface of APC. For instance, experiments carried out with the fifth component of the complement C5 as a model Ag showed that DC that are pulsed with low doses of C5 in thymic organ cultures are able to delete C5-specific transgenic T cells. Macrophages lacked this capacity but medullary epithelium was also able to delete T cells. These experiments, supported by others, suggest that DC together with epithelial medullary cells are pivotal in the induction of central tolerance [5–7].

A question remains: are all DC capable of inducing tolerance in the thymus or do thymic DC have special characteristics? The studies of Matzinger and Guerder [8] showed that allogenic DC from the spleen, when introduced into thymic organ cultures, were able to induce tolerance to alloantigens. Thus, a "professional" antigen-presenting cell from peripheral lymphoid tissue is able to activate peripheral T cells and also render thymocytes tolerant. These studies and some others [9] suggested that it is the developmental stage of the T cells which determines the negative outcome of Ag presentation rather than the nature of the DC. Additional evidence supports the notion that the threshold for tolerance induction in the thymus is lower than the threshold required by mature T cells for activation [10]. Thus, it is possible that specialized antigen-presenting cells are not required in the thymus for the induction of tolerance, except for the need of APC to efficiently capture, process and present self-Ag. Because of their efficient endocytic activity, due in part to the expression of specific receptors, and also to their ability to present exogenous Ag with MHC class I molecules (crosspresentation), it is possible that DC are crucial in the thymus for sampling the majority of self-Ag, and even to crosspresent self-Ag expressed in the thymic medullary epithelium [11].

Another important process during T-cell development is the generation of regulatory T cells, a type of suppressor cells. Growing evidence suggests an important role for these cells in the maintenance of peripheral tolerance. New evidence from Yong Jun Liu and coworkers indicates that thymic DC, when conditioned by thymic stromal lymphopoietin made by Hassall's corpuscles, play an important role in the generation of these thymic-born $CD4^+$ $CD25^+$ regulatory T cells from $CD4^+$ $CD25^-$ single positive thymocytes [12].

28.3 Dendritic Cells and Peripheral Tolerance

DC have been defined as the most potent professional antigen-presenting cells as well as "natural adjuvants" in the induction of antigen-specific immune responses

[13–16]. Surprisingly, they are also involved in the opposite outcome: the induction of peripheral tolerance [17–19]. Three questions arise:

What is the difference between "immune" DC and "tolerogenic" DC?

Are they the same cell type with distinct maturation states and phenotypes?

Conversely, are there distinct tolerogenic and immunogenic DC?

Some experimental evidence supports the idea for different maturation states between "resting" tolerogenic DC versus "activated" immunogenic DC. Others also suggest the presence of special DC for tolerance. However, an alternative point of view involves a common cell type phenotypically defined by the balance of positive versus negative signals at the time of the interaction between the DC and T cell.

Much like tolerance in the thymus, the sampling of exogenous Ag by APC in the periphery is a crucial point in the induction of specific immune tolerance. The capture of Ag by endocytic receptors on DC and their further presentation by MHC class II and class I molecules is essential; otherwise this process could be inefficient and not antigen-specific. DC express different groups of receptors: some of which are for capturing Ag; others are involved in increasing efficiency of presentation by class II molecules; and one last group is involved in Ag presentation by class I molecules. Ag presentation by both MHC pathways is crucial for tolerance induction in both $CD4^+$ and $CD8^+$ compartments. Three major groups of Ag receptors expressed by DC are involved in tolerance induction: (a) c-type lectins, which bind glycoproteins; (b) integrins, which contribute to the uptake of apoptotic cells at least *in vitro*; (c) Fc receptors that bind complexes of Ag with antibodies.

28.4 C-type Lectin Receptors

DC express a number of C-type lectin receptors (CLR) that contain single or multiple carbohydrate recognition domains. CLR bind structures on pathogens as well as self-glycoproteins, and this binding can be specific for the type of glycan and their organization patterns, thereby creating unique sets of carbohydrate recognition profiles. For instance, on DC, mannose receptors (MR) recognize single mannose moieties, whereas DC-SIGN has high affinity for more complex mannose residues in specific arrangements [20–22]. CLR have been shown to be involved in endocytosis for Ag presentation by class II molecules. This has been shown for MR (CD206), DEC-205 (CD205), DC-SIGN (CD209) and BDCA-2 [23–26]. Endocytosis by these receptors is mediated by conserved motifs in their cytoplasmic regions. Interestingly, after endocytosis, different intracellular routes are utilized for the different receptors. In bone-marrow DC, MR recycles from the early endosomes to the cell surface. By contrast, DEC-205 is able to further penetrate the endocytic pathway. This leads to a more efficient antigen presentation by class II molecules compared with MR [27].

Another important characteristic of the CLR is their ability to send intracellular signals at the time of the antigen capture. To date, cytoplasmic motifs with signaling capacities have been described in some of these receptors. DC-SIGN, the asialoglycoprotein receptor, Dectin-1, CLEC-1 and 2 contain cytoplasmic tyrosine residues that are part of the so-called immunoreceptor tyrosine-based activation motifs (ITAM) as well as the DC immunoreceptors (DCIR), which have potential inhibitory motifs (ITIM) [28, 29]. However, the role of these signaling motifs in the balance between tolerance and immunity has not been examined. In contrast, some other CLRs, including DEC-205 and BDCA-2, are devoid of signaling motifs.

Although CLRs are expressed by DC, some are also expressed by other cells. Thus, MR is expressed by macrophages but not by DC from the T-cell areas of the LN. Expression of most of these receptors is high in immature DC and decreases after maturation. An exception is DEC-205, which is expressed more by mature DC. In addition, some CLR are differentially expressed by certain subsets of DC.

28.4.1
Advantages of DEC-205 as an Endocytic Receptor for Antigen Presentation

Because of their characteristics, CLRs are interesting candidates to target Ag into DC. At this time, DEC-205 is the most widely studied receptor for targeting Ag into DC *in vivo*, while preserving their resting or steady state. The characterization of DEC-205 as a potent Ag receptor involved in antigen presentation expressed by DC but not by macrophages, and its lack of signaling motifs, are the factors backing this assertion. As the natural ligand of DEC-205 is still unknown, trials to target Ag into DC through this receptor have been carried out using mAb as a surrogate ligand. The first studies using this system involved the subcutaneous (s.c.) injection of the antibody, NLDC [30]. After injection of 10 µg of anti-DEC-205, most DC in the T-cell area of the LN were labeled. This was specific for DC as no label could be shown on T cells, B cells or macrophages [31]. This experiment was extended so that after harvesting different lymphoid tissues from mice injected s.c. with fluorescent anti-DEC-205, it was possible to see labels in ~60% DC from the draining LN. It was also possible to visualize DC from all LN including mediastinal, mesenteric and splenic nodes. This study showed the potential to load large numbers of DC systemically. After chemical conjugation of the Ag ovalbumin (OVA) with anti-DEC-205, it was also possible to detect the systemic distribution of the coupled Ag in DC [32].

The next set of experiments focused on the ability of this receptor to enhance antigen presentation *in vivo*. Thus, a MHC II restricted hen egg lysozyme (HEL) peptide expressed as a fusion protein with the heavy chain of anti-DEC-205 required only a few nanograms to induce efficient class II presentation as revealed by the proliferation of HEL antigen-specific 3A9 transgenic $CD4^+$ T cells [31]. After chemical coupling of OVA with anti-DEC-205, it was also possible to see proliferation of OVA-specific transgenic $CD4^+$ T cells and OVA-specific $CD8^+$ T cells. These studies indicated the potential of this receptor to mediate presentation on both MHC class II and, interestingly, class I (crosspresentation). In the case of OVA, the

increase in the efficiency of Ag presentation, compared with the uncoupled Ag , was 500 times for MHC class II and 10 000 times for MHC class I. This antigen presentation *in vivo* was mediated by DC since only $CD11c^+$ cells were capable of inducing proliferation of specific T cells *in vitro* after *in vivo* injection of the Ab OVA conjugates. The ability to present the Ag by DC was also systemic, as DC taken from distal lymph nodes and from the spleen induced Ag-specific T-cell proliferation after s.c. injection [33].

28.4.2
DEC-205: an Endocytic Receptor that Preserves the Steady State in the DC after the Capture of the Antigen

The next experiments using the DEC-205 model to target Ag to DC *in situ* were designed to determine the DC phenotype after targeting by Ab alone or conjugated with the Ag. The delivery through anti-DEC-205 did not change the phenotype of the DC, suggesting that after ligation the DEC-205 receptor is not able to send a maturation signal [31, 33]. Similar results were achieved in the presence of antigen-specific T cells. The fact that after Ag targeting, DC do not undergo any apparent change creates the opportunity to use this model to evaluate the role of resting DC in the induction of peripheral tolerance.

28.5
Induction of Peripheral Tolerance by Resting Dendritic Cells

Experimentally, tolerance is defined as antigen-specific nonresponsiveness to a challenge with Ag in the presence of potent adjuvants. Strong experimental evidence suggests the participation of DC in the induction of peripheral tolerance [34–39]. The first experiments to evaluate the consequences of Ag delivery specifically to resting DC *in situ* were made by Hawiger and coworkers. After $CD45.1^+$ $CD4^+$ transgenic HEL-specific T cells were adoptively transferred into a mouse injected with anti-DEC-205 HEL peptide, it was possible to see a vigorous proliferation of antigen-specific T cells. The proliferation was comparable to that of mice injected with the HEL peptide in CFA. HEL-specific T cells were not detected 7–14 - days after the initial injection with anti-DEC-HEL. More importantly, at day 7, these cells were unable to respond to the challenge with the HEL peptide in CFA [31]. Although these results show that resting DC are efficient antigen-presenting cells, able to induce strong T-cell proliferation, the final consequence of this initial antigen presentation was antigen-specific unresponsiveness. These studies were confirmed using OVA conjugated with anti-DEC-205. In this case the tracked cells were OVA-specific $CD8^+$ T cells. Thus, Ag taken up by DEC-205 can induce tolerance also in the $CD8^+$ compartment [33]. In both systems, the mechanism proposed for tolerance was clonal deletion, as it was not possible to detect the relevant T cells 7–14 days post-injection even in nonlymphoid tissues.

28.5.1
The Same Dendritic Cells Could Operate in the Induction of Immunity

One important point to clarify is whether the DC involved in the induction of tolerance were also able to induce immunity and reverse the tolerance upon a maturation stimulus. After coinjection of an agonistic Ab against CD40, it was possible to induce a strong CD4 response after injection of anti-DEC-205 HEL [31]. Moreover, a combined CD4 and CD8 immune response, including IFNγ release and cytolytic $CD8^+$ T cells was induced after the injection of anti-DEC-OVA [33]. Cumulatively, these results show that resting or steady state DC are able to induce peripheral tolerance and suggest that the same DC, depending on their status or active phenotype, can also induce an immune response (Fig. 28.1).

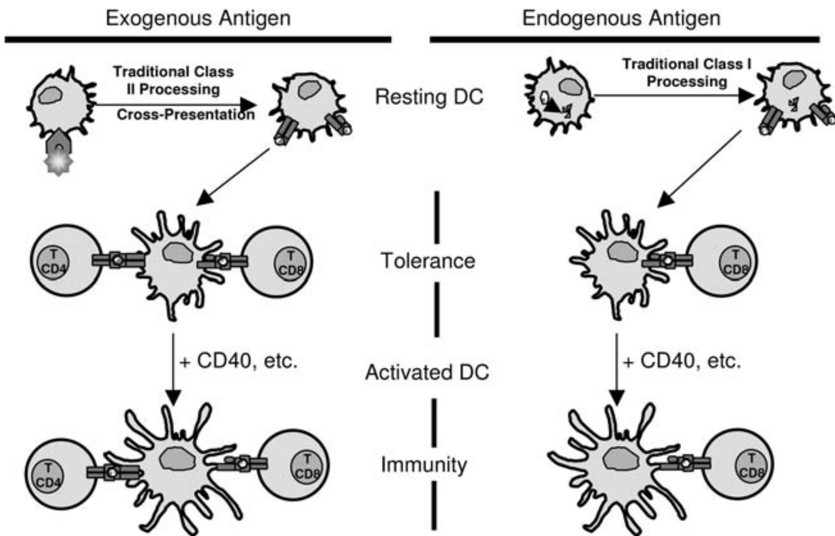

Fig. 28.1 Resting versus activated DC in the induction of tolerance or immunity. Endogenous or exogenous Ag are processed and presented, in the context of class I and class II molecules, by resting DC. The consequence of this antigen presentation is the induction of antigen-specific tolerance in the CD4 and CD8 compartments, accordingly. Activation of DC by agonistic anti-CD40 leads to the induction of immune responses.

28.5.2
The Induction of Tolerance by Steady-state Dendritic Cells Promotes Avoidance of the Induction of Autoimmunity

The next question, when using the anti-DEC-205 targeting system, is how deep is the induced tolerance? Is it sufficient to protect from autoimmune disease? Nussenzweig and coworkers showed, by using the acute EAE model, that tolerance induced after injection of just 15 _g of encephalitogenic oligodendrocyte glycoprotein (MOG) peptide coupled to anti-DEC-205 was sufficient to protect mice from

autoimmune disease [40]. After examination of the spinal cord of treated and control mice an increased numbers of CD4+ T cells could be identified in spinal cord of mice that show symptoms of EAE, no such cells were found in spinal cords of mice that had been pre-treated with anti-DEC-205 MOG peptide. Thus targeting resting DC with MOG peptide prevents accumulation of effector T cells in the nervous system preventing the induction of EAE.

28.5.3 Surface Molecules are Involved in Peripheral Tolerance Induction by Resting Dendritic Cells through DEC-205

As previously mentioned, in the first two systems using anti-DEC HEL peptide or anti-DEC coupled with OVA, the proposed mechanism for tolerance was deletion because of the inability to find the adoptively transferred antigen-specific T cells 7–14 days after the induction of tolerance. Interestingly, in the system using anti-DEC-205 MOG peptide, antigen-specific T cells remain in the mice 3–7 days after injection. These cells were not fully anergic since they remain responsive to T-cell receptor stimulation *in vitro* but they failed to respond to Ag *in vivo* [40]. The *in vivo* phenotype of unresponsive T cells was similar to responsive cells in the expression of all the classical activation markers such as CD69, CD25 CD44 and CD62L. The only difference was the expression of CD5 by tolerant T cells, which is an inducible negative regulator during selection of thymocytes. Additional experiments suggested that CD5 was involved in the induction of tolerance after injection of anti-DEC MOG. Therefore, assuming that the same resting DC are involved in the presentation of all the Ag coupled to anti-DEC-205, the question is: how can the same DC induce different tolerance mechanisms? One possibility is that the affinity of the TCR at the time of the interaction dictates the final outcome.

28.5.4 Additional Evidence Supports the Role of Resting DC in the Induction of Peripheral Tolerance

Additional studies by Probst et al. support the hypothesis that the status of DC determines the induction of peripheral tolerance versus immunity. The system used was an inducible transgenic model, which permits the expression and further Ag presentation of peptides from lymphocytic choriomeningitis virus (LCMV) exclusively by resting DC. After presentation of LCMV peptides in the absence of infection, specific peripheral tolerance was observed. The tolerance was deep because it could not be overcome by a subsequent infection with LCMV [41]. These experiments were carried out without adoptively transferring T cells. In this system, the Ag is expressed in the cytosol and it is presented by the classical endogenous pathway by MHC class I molecules. Approximately 5% of DC express the Ag without restriction to a special subset, suggesting that this conclusion can be applied to practically any DC. On the other hand, antigen presentation of the LCMV peptides by activated DC with the agonist anti CD40 Ab leads to the induction of protective

immunity, providing further evidence that the same cells can participate in the induction of tolerance or immunity (Fig. 28.1).

28.6 Surface Molecules Involved in the Induction of Peripheral Tolerance

Although it is clear that steady-state DC are involved in the induction of peripheral tolerance, more information is needed about the surface molecules on DC and T cells that are important for the initial proliferation and activation but also for the final disappearance or functional inactivation of the T cells. One simple explanation could be the difference in the level of co-stimulatory molecules expressed by resting versus activated DC. Considering that resting DC are immature or semi-immature with low expression of co-stimulatory molecules compared with mature or activated DC, it is possible to explain the difference in outcome after T-cell interaction by the classical second-signal dogma. However, the lack of a second signal from the traditional co-stimulatory molecules does not necessarily lead to the induction of peripheral tolerance even in the absence of co-stimulation by the B7-CD28 pathway. When stimulated solely though the TCR, CD4 T cells respond poorly but the surviving cells are not tolerant, and respond like naïve lymphocytes [42], which supports the notion that it is not the lack of co-stimulatory molecules, but the presence of some other trigger, able to interact with T lymphocytes, which sends negative signals leading to the induction of tolerance rather than immunity.

In T cells, the cytotoxic T lymphocyte associated CTLA-4 or CD152 has been shown to participate in self-tolerance since CTLA-4 deficient mice develop lymphoproliferation and lethal autoimmunity [43]. This suggests its participation in the maintenance of T-cell tolerance *in vivo* [44]. The ligands described for CTLA-4 are CD80 and CD86 expressed by DC. Recent evidence suggests that CD80 rather than CD86 acts as the main ligand for CTLA-4 [45, 46]. Another molecule involved in the negative control of T-cell responses is the protein, programmed cell death (PD-1). Mice deficient in this molecule also develop autoimmune disorders [47, 48]. The ligands for PD-1 are B7-H1 and B7-DC. Based on their characteristics, both CTLA-4 and PD-1 are appropriate candidates to participate in tolerance induction. Recently, additional experiments by Probst using the inducible transgenic model for the expression of LCMV peptides on resting DC showed that tolerance induction depends on signaling through the inhibitory receptor, PD-1 [49]. Blocking of CTLA-4 also resulted in impaired tolerance. Interestingly, PD-1 and CTLA-4 appear to act synergistically, as the effect on tolerance induction was more dramatic in the absence of both interactions. This is the first evidence of the involvement of these molecules in the induction of peripheral tolerance by resting DC. However; these results need to be expanded to other systems to be considered as a general mechanism since blocking of CTLA-4 during tolerance induction by anti-DEC-205 OVA injection did not impair the tolerance outcome (Bonifaz, L. unpublished data). The role of inhibitory receptors in the induction of tolerance supports the idea of a balance of negative versus positive signals at the time of the DC–T cell interaction for the induction of tolerance versus immunity (Fig. 28.2a).

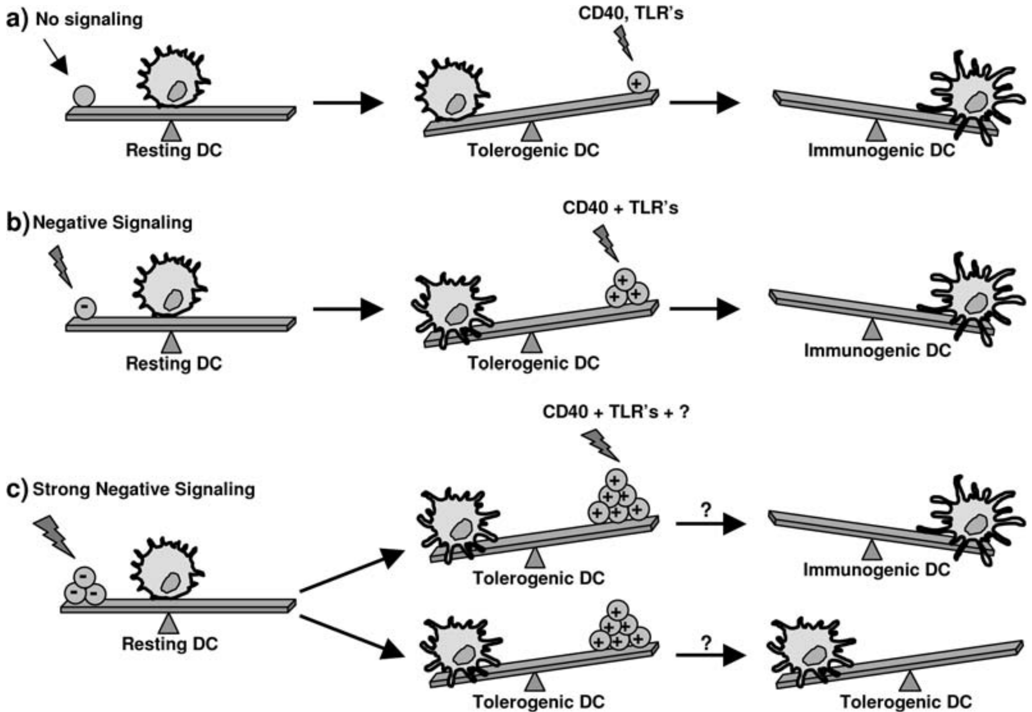

Fig. 28.2 Balance of negative versus positive signals in the induction of tolerogenic or immunogenic DC. (a) Endogenous constitutive antigen presentation or presentation after endocytosis in absence of signaling (i.e. via DEC-205) preserves the resting DC phenotype. This tolerogenic phenotype can be reversed to an immunogenic phenotype by positive signals (CD40, TLR ligands, etc). (b) The tolerogenic phenotype can be strengthened by endocytosis via receptors with ITIM motifs. To be reversed, this phenotype may need stronger signaling or an accumulation of positive signals. (c) The presence of strong negative signals could induce expression of inhibitory molecules (ILT-3, ILT-4) that lead to a block of the NFκB pathway. This strong tolerogenic phenotype could be irreversible or reversed only by powerful (?) positive signals.

28.7 Other Receptors Involved in the Induction of Tolerance that can Preserve the Resting of DC or Induce Negative Signaling

28.7.1 Integrins

Integrins are a family of transmembrane receptors in which distinct α and β units are combined resulting in individual ligand specificity. Recently it was described a role for integrins in the uptake of cellular debris and apoptotic material by DC, which is carried out through different receptors such as thrombospondin receptor (CD36), αvb3 and αvb5 receptors, the complement receptors 3 and 4 [50, 51] and

others [52]. Therefore, *in vivo*, DC constantly transport apoptotic self-Ag from tissue sites to the T-cell areas of draining LN and present peptide–MHC complexes to T cells [53, 54]. However, this presentation does not lead to the induction of an immune response. In fact, there is strong evidence supporting the induction of tolerance as a safeguard mechanism to avoid autoimmunity [55, 56].

Uptake of dead cells by complement receptors does not lead to DC activation, thus further antigen presentation would be in a tolerogenic context [57]. It has also been reported that uptake through CD36 integrin blocks DC maturation [58]. In addition, uptake of apoptotic cells by CD11b/CD18 integrin inhibits production of pro-inflammatory cytokines [59]. The role of integrins in the induction of tolerance is extensively reviewed in Chapter 29. Nevertheless, the findings mentioned herein are additional examples that the endocytosis of apoptotic material either sends negative signals to ensure the induction of peripheral tolerance or does not alter the resting state of DC, thus not inducing immunity to such material (Fig. 28.2b).

28.7.2
Fc Receptors

Fc receptors comprise a family of proteins capable of interacting with the constant region of immunoglobulins. Fc receptors are not expressed exclusively by DC and have been extensively studied in B cells. These receptors have an important role in both efficient endocytosis of antigen–antibody complexes as well as in efficient antigen presentation by class II and class I molecules [60–62]. The role of these receptors in the induction of an efficient immune response has been extensively studied. However, because of the existence of inhibitory Fc receptors containing ITM motifs, a role for FcγR in the induction of tolerance also needs to be considered. The physiological consequences of cell-bound IgG immune complexes are modulated by a balance between activating and inhibitory Fc gamma receptors [63, 64]. Studies by Kalergis and coworkers showed that targeting immune complexes to DC from mice genetically lacking inhibitory FcγRIIb leads to enhanced generation of antigen-specific $CD8^+$ T-cell immunity *in vivo* [65]. Genetic deletion of FCγRIIb leads to spontaneous autoimmunity in mice [66]. Recently, an interesting study by Dhopakar and coworkers showed the role on inhibitory Fc receptors in maintaining human DC in an immature state. Selective blockade of this inhibitor receptor enables DC maturation leading to a protective immune response [67]. Therefore, these kinds of receptors, involved in both endocytosis and processing, are another clear example of a balance of inhibitory and activator signals responsible for the final outcome after T-cell proliferation and activation (Fig. 28.2b).

28.7.3
Suppressor and Regulatory T Cells

Another important tolerance mechanism to avoid immune response against self- and innocuous Ag is the existence of regulatory cell populations. At least two pop-

ulations of T cells with regulatory functions have been described. The first one is generated in the thymus during T-cell development and are called suppressor cells with the $CD4^+$ $CD25^+$ phenotype. The second population of regulatory cells, called Tr1, can be generated and expanded in the periphery from CD25– precursors. The function of these cells is to block the function of other effector T cells. Both populations have been implicated in suppressing the immune response *in vivo* [68, 69]. The inhibition of the immune response by regulatory T cells is believed to be central to the prevention of autoimmune diseases [70, 71]. The mechanism of action of both populations of regulatory cells appears to be distinct. Thus, after TCR-triggering, that is MHC-restricted, $CD4^+$ $CD25^+$ regulatory cells (Tr) inhibited the immune response through an Ag and MHC restricted independent mechanism. These cells, which are apparently anergic, express the CTLA-4 molecule, which appears to play an important role in the regulatory process. On the other hand, Tr1 cells suppress the immune response in an antigen-specific manner by a mechanism dependent on cytokines such as IL-10 and TGFβ.

Immature DC have been implicated in the generation and expansion of Tr1. The studies by Jonuleit and coworkers [72] and Dhodapkar and coworkers [73, 74] showed participation of immature DC in the induction of human Tr1-like cells. Interestingly, experiments by Yamazaki and coworkers [75] showed direct expansion of functional $CD25^+$ $CD4^+$ regulatory T cells by DC. Such expansion could be achieved in an antigen-specific manner. $CD25^+$ $CD4^+$ T cells proliferated extensively in response to steady state or mature DC . The induction of these regulatory T cells by DC can suppress autoimmune diabetes [76]. These findings challenge some of the previous models of Tr function and emphasize the role of DC in the maintenance of tolerance by the induction of regulatory T cells.

28.8 Notch Ligands as Surface Molecules Involved in the Induction of Regulatory T Cells

Notch signaling is an evolutionary pathway used to direct developmental cell fate decisions in multiple organs [77]. Notch signaling is initiated through ligand–receptor interactions, leading to proteolytic cleavage of the receptor, a process that liberates the cytoplasmic domain of Notch. Notch encodes an evolutionary conserved transmenbrane receptor activated by two distinct cell surface ligands called Delta and Serrate. Mammals have four receptors (Nothc 1-4) and five ligands: Jagged1 and Jagged 2 (homologs of Serrate) and Delta 1, Delta 3 and Delta 4 (homologs of Delta). In the immune system the Notch pathway is involved at different levels. Early during T-cell ontogeny in the thymus, Nocht 1 signaling is necessary and sufficient for T-cell lineage commitment [78].

The identification of Notch receptor on peripheral T cells and its ligands on APC suggested their participation in peripheral T-cell decisions. Recent evidence suggests the participation of the Notch pathway in the differentiation of naïve T cells into regulatory T cells. Constitutive over-expression of Jagged 1 on murine dendritic cells induces differentiation of T cells into long-lived Tr1 cells, which can trans-

fer tolerance to naïve mice [79]. In addition transgenic mice expressing Notch3 intracellular domain under control of the proximal Lck promoter have increased regulatory T-cell function, as shown by a higher degree of protection than control mice in a model of inducible autoimmune diabetes [80]. Regulatory T cells have higher Notch expression than $CD4^+CD25^-$ conventional T cells, raising the possibility that signaling through the Notch 3 receptor regulate the development, expansion and function of $CD4^+CD25^+$ regulatory T cells. Finally, priming of naïve human T cells in the presence of Jagged 1 expressing APC lead to reduced proliferation and partial inhibition of the cytotoxic effector function of CD8 T cells through the induction of regulatory T cells [81, 82].

Although DC and other APCs can express several Notch ligands [79], induction of regulatory T cells via Notch has not yet been formally demonstrated. Studies on the role of the Notch pathway in the terminal differentiation of DC showed that Notch signaling is necessary but not sufficient for their final differentiation. Interestingly, in the presence of Notch signaling, DC keep an immature phenotype [83]. Therefore one possibility for DC to induce regulatory T cells via Notch is the inability of DC to mature, hence promoting the induction of regulatory T cells as has been reported. Another possibility is that Notch ligands could be differentially induced on DC under different stimuli, as has been shown for Th1 and Th2-type T cells [84]. After NotchL expression, DC could acquire the potential to induce regulatory T cells. Even though this field is at its beginning, Notch ligands on DC and Notch receptor in T cells are good candidates to participate in the decision of tolerance versus immunity through the induction of regulatory T cells.

28.9
ILT-3 and ILT-4: Two Inhibitory Molecules Involved in Tolerance Induction

A third population of regulatory CD8 T cells has been described in humans. These cells have the noncommon phenotype $CD8^+$ CD28– T cells and have been also referred to as suppressor T (Ts) cells. Ts cells can be generated *in vitro* after multiple rounds of stimulation of human peripheral blood mononuclear cells with either allogenic or xenogenic donor APCs [85, 86]. Ts $CD8^+$ cells are MHC class I restricted. They can suppress the response of $CD4^+$ T cells in an antigen-specific manner through a mechanism dependent on the presence of dendritic cells as bridge. After Ag presentation by either an immature dendritic cell or a monocyte, Ts cells induce in the APC the expression of two molecules: immunoglobulin-like transcript 3 and 4 (ILT3 and ILT4). These molecules have been described by Colona and are involved in negative signaling by the presence of ITIMs in their cytoplasmic tails [87]. Interestingly, the interaction of Ts cells with DC results in inhibition of the NFkB pathway and in downregulation of the B7 co-stimulatory molecules [88]. As a consequence of this interaction, the resulting DC is a tolerogenic DC able to induce unresponsiveness of $CD4^+$ T cells (Fig. 28.2c). After Ag presentation by these tolerogenic DC, $CD4^+$ T cells become anergic and this state could be overcome by

the addition of IL-2. The relevance of this regulatory population *in vivo* has been documented through the isolation of $CD8^+$ CD28– suppressor T cells from transplant patients who did not undergo acute rejection [89]. Ts cells isolated from patients were shown *in vitro* to induce the upregulation of ILT3/ILT4 on MHC-matched APCs. In addition ILT-3 and ILT-4 have been detected on APC from transplant donors *in vivo* [90].

The generation of a DC with a special phenotype expressing specific inhibitory molecules and with impaired signaling through the NF_B pathway could support the notion of the existence of a special tolerogenic dendritic cell. The questions are: Do DC with this phenotype exist under steady state conditions? If not, under which conditions can they be generated? Are these DC also able to induce an immune response under strong inflammatory conditions? Alternatively, after their induction, is their only function the induction of tolerance? These questions still require investigation but some new evidence suggests the existence of DC with regulatory–tolerogenic functions.

28.10
Special DC for Tolerance?

The existence of specialized DC for tolerance induction has been assumed for a long time as an easy explanation for the induction of tolerance versus immunity. Initial *in vitro* studies by Shortman and coworkers [91], demonstrated that splenic CD8– DC induced a strong proliferative $CD4^+$ T-cell response, whereas $CD8^+$ DC induced a lesser response. Another report showed that $CD8^+$ DC downregulated the secretion of IL-2 by $CD8^+$ T cells [92]. These findings led to the hypothesis that $CD8^+$ DC are a subset preferentially involved in the induction of tolerance than in the induction of immunity. However, numerous reports have shown that $CD8^+$ DC are the major producers of IL-12 [93, 94]. Moreover, several reports have shown the participation of this subset in the induction of a potent immune response including IFNγ-producing T cells. To date, is clear that $CD8^+$ DC are able to induce both tolerance and immunity [95]. It is possible that this particular subset of DC has a more tolerogenic phenotype compare with the $CD8^-$. As an example, a $CD11c^+$ $CD8^+$ subset of DC both in mice and humans express the inducible enzyme indoleamine 2,3-dioxygenase (IDO) that may be involved in a novel suppression mechanism of T-cell proliferation [96, 97]. In addition, $CD8^+$ cells could have a high expression of ligands involved in the interaction with inhibitory receptors on T cells, such as the PD-1 ligands or CD80 that interact with CTLA-4. Another possibility is the role of $CD8^+$ DC in the endocytosis of apoptotic material, during which negative signaling could occur. Therefore, it is possible that strong positive signals are required to induce immune responses mediated by these cells (Fig. 28.2b).

28.11
Regulatory–tolerogenic DC

Is spite of the strong evidence supporting the notion that DC are capable of inducing tolerance or immune response depending on their activation status, the existence of DC with toleregenic phenotype has recently been reported, which have been nicknamed "regulatory DC" for their ability to differentiate naïve T cells into regulatory cells. Initial evidence for the generation of DC with regulatory–tolerogenic functions emerged from *in vitro* cultures of bone-marrow cells, and has been further supported by *in vivo* identification of similar populations. Wakkach and coworkers showed the presence of DC with an immature phenotype and with low expression of CD11c and high expression of CD45RB in cultures in the presence of IL-10 [98]. These cells developed *in vitro* in the presence of IL-10 but could also be identified *in vivo* in the spleen and lymph nodes of normal mice and are increased in the spleens of IL-10 Tg mice. These DC induce tolerance though the differentiation of Tr1 both *in vitro* and *in vivo*.

Studies by Zhang and coworkers [99] showed the potential of stromal cells to induce differentiation of DC into regulatory DC with the ability to suppress T-cell responses. Svensson and coworkers [100] found that DC could be differentiated into regulatory DC in the presence of stromal cells. These DC also had the ability to suppress T-cell responses and can also be identified *in vivo*, especially after infection with *Leishmania donovani*, where a more effective differentiation of this highly potent population of regulatory DC was observed.

Populations of regulatory DC with the ability to secrete IL-10 but not IL-12 have also been identified in the airways and in the intestinal tract [101, 102], where the balance between immune response and tolerance has to be tipped toward tolerance to prevent extensive permanent inflammation. These initial findings suggest that both in the steady state and after induction it is possible to differentiate an *in vivo* population of DC with a strong tolerogenic potential. As attractive as it seems, this model does not rule out the possibility that these findings might only reflect a functional status of a cell with otherwise immunogenic potential (Fig. 28.2c).

28.12
Concluding Remarks

Peripheral tolerance mediated by DC is still a field under extensive investigation, with many questions remaining unanswered. However, the same DC might play a crucial role both in the induction of a deep peripheral tolerance and in the induction of efficient immune responses depending on the cell's activation state. It is quite possible that the balance of negative versus positive signals sent at the time of the T cell–DC interaction is the key for the induction of tolerance versus immune responses. This balance can be achieved by the expression of certain surface molecules involved in Ag capture and presentation that either preserve the steady state of the DC or send negative signals to induce a more tolerogenic DC pheno-

type. The mechanism involved in the induction of tolerance versus immune response might also be dependent on the surface molecules expressed by resting DC or by surface molecules expressed as a consequence of negative signaling. The main question yet to be answered is whether tolerogenic DC are always capable of becoming immunogenic DC. In any case, the study of surface molecules involved in the induction of tolerance versus immunity is a broad field of investigation that will lead to a better understanding of the process as well as possible experimental manipulations in the future.

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29
Interaction Between Dendritic Cells and Apoptotic Cells

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29.1
Introduction

Apoptosis, or programmed cell death, constitutes a series of genetically-controlled, energy-dependent cellular events by which cells actively orchestrate their own demise. During this process, cells undergo biochemical and morphological changes, including redistribution of lipids of the plasma membrane, loss of mitochondrial membrane potential, inter-nucleosomal DNA cleavage, fragmentation of the nucleus and cytoplasm into membrane-enclosed apoptotic bodies, and shedding of apoptotic blebs from the cell surface [1]. The changes in the molecular composition of the surface membrane of cells undergoing apoptosis initiates the recognition and removal of apoptotic cells by professional phagocytes including macrophages and immature dendritic cells (DC) and by neighboring parenchymal or stromal cells (e.g. fibroblasts, epithelial cells, endothelial cells, glomerular mesangial cells, etc) able to function as semi-professional phagocytes.

Clearance of apoptotic cells is an essential process during embryogenesis, morphogenesis and maintenance of tissue homeostasis. In physiological conditions, apoptotic cells are phagocytosed rapidly, while their plasma membrane retains its integrity, before leakage of toxic mediators like proteases, cationic proteins and oxidizing molecules into the extracellular space. Thus, the rapid clearance of cells in early apoptosis prevents the potential tissue damage resulting from cells that disintegrate by late apoptosis or secondary necrosis. Moreover, release of proteolytic fragments of self-proteins and oligonucleosomal DNA to the extracellular space may trigger an autoimmune response. In this regard, it is known that apoptotic cells externalize and concentrate nuclear autoantigens on the surface of apoptotic blebs that are shed to the extracellular milieu [2].

In physiological conditions, the clearance of cells in early apoptosis is so efficient that phagocytes with internalized apoptotic cells are only detected histologically in tissues undergoing a high rate of apoptosis, such as thymus, bone marrow (BM) and germinal centers of B cell follicles. The speed and efficiency of removal of apoptotic cells in the steady state is probably one of the reasons by which this type of

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cell death was not identified until recently [3, 4]. More recent data indicate that apoptotic cells may actively suppress inflammation and participate in induction/maintenance of T and B cell peripheral tolerance. This phenomenon is mediated by a highly specialized interaction between apoptotic cells and phagocytes of the innate and adaptive immune system. However, depending on the characteristics of the apoptotic cells and the mediators present in the extracellular milieu, apoptotic cells may eventually trigger inflammation and promote immunity [5].

29.2
Dendritic Cells Phagocytose and Process Apoptotic Cells

In 1986, the studies of Fossum and Rolstad [6] on allogeneic lymphocyte cytotoxicity demonstrated for the first time that interdigitating DC of lymph nodes and spleen were able to internalize fragments derived from allogeneic lymphocytes killed by host natural killer cells. However, the conclusive evidence that DC phagocytose apoptotic cells was obtained later from studies on Langerhans cells (LC) in the vaginal epithelium of rodents. By means of electron microscopy, Parr and colleagues [7] demonstrated that LC of the vaginal epithelium internalized apoptotic epithelial cells during late metestrus and early diestrus in mice. However, the physiological importance of this observation remained unclear until 1998 when Albert et al. [8] demonstrated *in vitro* that apoptotic cell-derived antigens (Ag) are acquired by human BM-derived DC and presented in MHC class-I molecules to $CD8^+$ cytotoxic T lymphocytes (CTL). Presentation of peptides derived from extracellular Ag via MHC class-I molecules to $CD8^+$ T cells is known as "cross-priming". This pathway of Ag processing allows $CD8^+$ T cells to recognize peptides that do not have access to the cytosol of Ag presenting cells (APC) and therefore, that are unable to follow the endogenous pathway of antigenic processing. Albert et al. [8] also demonstrated that, although macrophages were more efficient at phagocytosis of apoptotic cells than DC, only DC efficiently crosspresented apoptotic cell-derived Ag to $CD8^+$ T cells. A similar observation was made in a different experimental model where DC, but not macrophages, crosspresented Ag from *Salmonella*-infected apoptotic cells via MHC class-I molecules to specific $CD8^+$ T cells [9]. Furthermore, *in vivo* studies have shown that unlike macrophages, DC pulsed with apoptotic cells induce the generation of antitumor CTL [10]. Addition of macrophages to DC cultures impaired the ability of DC to crosspresent apoptotic cell-derived peptides [8]. It is likely that, due to their high phagocytic ability, macrophages sequester (and degrade completely) apoptotic cells from DC [8]. Unlike monocyte/macrophages, the ability of DC to crosspresent internalized Ag is in part due to a unique membrane transport pathway. This mechanism allows internalized Ag to gain access to the cytosolic Ag-processing machinery and to the conventional MHC class-I molecule presentation pathway [11].

Alternatively, DC can process apoptotic cell fragments on MHC class-II molecules with high efficiency for presentation to $CD4^+$ T cells by using the endocytic pathway of antigenic processing. Inaba et al. [12] have shown that murine BM-de-

rived DC present Ag from internalized apoptotic cells to CD4$^+$ T cells 1–10000 times better that preprocessed peptide.

Immature DC phagocytose apoptotic cell fragments more efficiently than mature DC [13]. This phenomenon requires the presence of extracellular calcium and rearrangement of the DC cytoskeleton, as it is inhibited by incubation with EDTA or cytochalasin D [13, 14]. The $\alpha_v\beta_5$ integrin not only mediates the binding of apoptotic cells to DC but also their internalization. Engagement of the apoptotic cell to the $\alpha_v\beta_5$ heterodimer on the phagocyte surface results in the recruitment of the CrkII-Dock180 molecular complex, which triggers Rac1 activation and phagosome formation [15–17].

Once internalized by DC, apoptotic cells are processed within MHC class-II-rich compartments (MHC-II$^+$ LAMP$^+$ H-2M$^+$ vesicles) [12]. Incubation of DC with ammonium chloride, which inhibits acidification and proteolysis within endocytic vacuoles, blocked presentation of apoptotic cell-derived peptides through MHC class-I and II [8, 12]. The intracellular route by which apoptotic cell-derived peptides generated within endocytic vacuoles of DC gain access to the lumen of the endoplasmic reticulum to be loaded into MHC class-I molecules for crosspresentation remains unclear. The fact that lactacystin, a 26S proteasome inhibitor, partially blocked crosspresentation of apoptotic cell-derived peptides by DC indicates that both classical and nonclassical MHC class-I pathways participate in this process [8].

29.3 The Phagocytic Synapse

A critical feature of apoptotic cells is their ability to be recognized specifically and removed by professional phagocytes (e.g. macrophages, immature DC) or by neighboring parenchymal or stromal cells. This mechanism of recognition and uptake of apoptotic cells is very complex and has not been fully characterized. Although there is extensive literature regarding the receptors used by macrophages for phagocytosis of apoptotic cells, little is known about the mechanisms employed by DC.

The surface of apoptotic cells displays a series of Apoptotic-Cell-Associated Molecular Patterns (ACAMP) that function as “eat me” signals that interact with Pattern-Recognition Receptors (PRR) expressed on the surface of phagocytes [18]. In general, these eat me signals do not require de novo protein synthesis by dying cells, instead they are generated by modification or translocation of preexisting plasma membrane components or by deposition of soluble extracellular molecules (opsonins) on the surface of apoptotic cells. Fadok et al. [19] have coined the term “phagocytic synapse” for this highly sophisticated molecular interaction between phagocytes and apoptotic cells.

During the last decade, numerous ACAMP and their corresponding PRR have been identified. Some of them are ligand–receptor pairs composed of receptors expressed by the phagocytes that directly bind ligands present on the apoptotic cell

surface (Fig. 29.1A). Other receptors require the recruitment of "bridging molecules" from the extracellular fluid that function as opsonins, linking ligands on the surface of apoptotic cells with PRR expressed by the phagocytes (Fig. 29.1B). The following are some of the most extensively studied ligand–receptor interactions that participate in phagocytosis of apoptotic cells by phagocytes of the immune system.

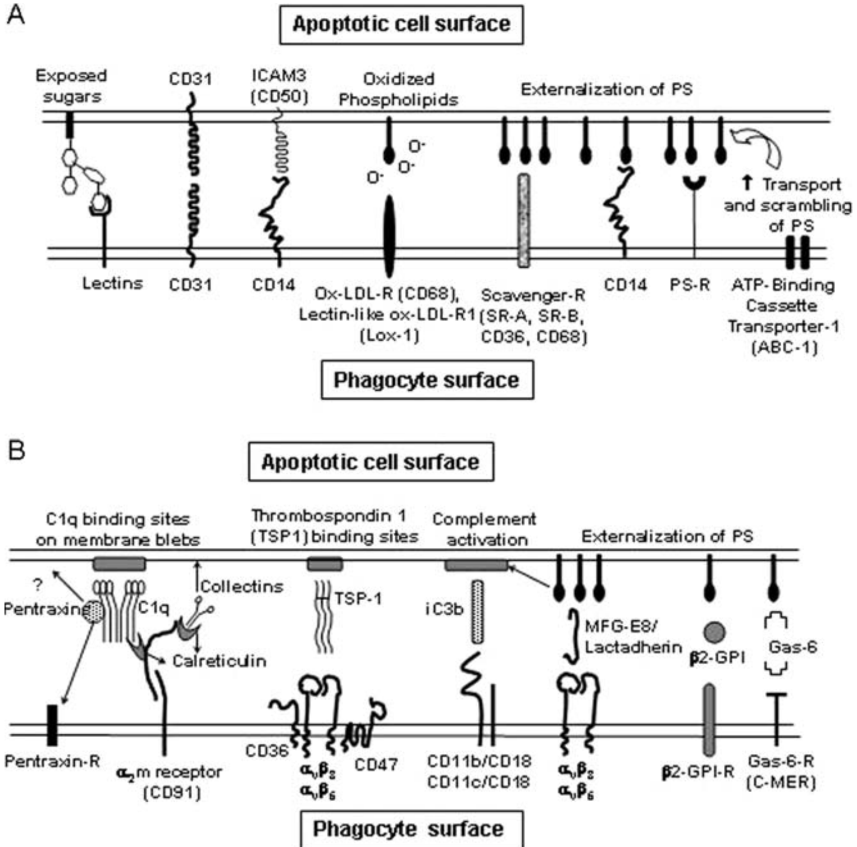

Fig. 29.1 Ligands and receptors involved in clearance of apoptotic cells by phagocytes. (A) Pattern-recognition receptors expressed by phagocytes and their ligands on the apoptotic cell surface. (B) Bridging molecules that participate in recognition, attachment and internalization of apoptotic cells by phagocytes including DC. PS: phosphatidylserine; Ox-LDL: Oxidized low density lipoprotein; -R: receptor; MFG: milk-fat globule protein; β2-GPI: β2 Glycoprotein I; Gas-6: growth arrest specific gene-6 product; C-MER: MER tyrosine kinase receptor; α2m: α2 macroglobulin.

29.3.1 Externalized Phosphatidylserine (PS) and Receptors for PS

One of the earliest changes in apoptotic cells is the loss of phospholipid asymmetry of the plasma membrane caused by the translocation of PS, phosphatidylethanolamine (PE) and phosphatidylcholine to the outer leaflet of the plasma membrane [20]. Externalization of PS occurs hours before the characteristic morphological changes of apoptosis. In viable cells, an ATP- and magnesium-dependent aminophospholipid translocase flips PS, and to a lesser extent PE, from the outer to the inner leaflet of the plasma membrane maintaining the phospholipid symmetry of the membrane [19]. On the other hand, a phospholipid scramblase moves phospholipids bidirectionally across the plasma membrane [19]. The entry of calcium that occurs during the initial stages of apoptosis inhibits the lipid translocase and activates a lipid scramblase resulting in the accumulation of PS on outer leaflet of the plasma membrane. Phospholipid scrambling seems to be facilitated by the ABC-1 transporter, a member of the ATP-binding cassette superfamily of membrane transporters. There is evidence that the facilitating effect of ABC-1 on clearance of apoptotic cells depends on the effects of ABC-1 transporter on the phospholipid asymmetry in both the phagocyte and target apoptotic cell [21, 22].

Binding of externalized PS with fluorochrome-conjugated annexin V has been a very useful tool to detect cells in early and late apoptosis by microscopy and flow cytometry [20]. The presence of PS on the surface of apoptotic cells is recognized by phagocytes via different receptors, including a stereospecific PS receptor (PS-R) [20, 23]. Recent evidence indicates that apoptotic cells also externalize annexin I (also known as lipocortin), a molecule that probably facilitates the interaction of the extremely small polar group of PS with the PS-R [24]. Mice lacking PS-R exhibited a severe deficiency in the clearance of apoptotic cells [25]. Several scavenger receptors (SR) (e.g. SR-A, SR-B1, CD36 and CD68), CD14 and soluble opsonins may also bind externalized PS and trigger internalization of apoptotic cells by phagocytes (Fig. 29.1). Most of the receptors for PS have been studied in macrophages, and little is known regarding their function during internalization of apoptotic cells by DC. Murine splenic DC transcribe mRNA for PS-R, SR-AII, SR-BI, CD14 and CD68 [26]. Whether DC translate all these mRNA transcripts into functional receptors able to recognize PS on apoptotic cells is still unknown, although there is evidence that DC express the stereospecific PS-R [19]. The presence of receptors for PS on the surface of DC is supported by the fact that human monocyte-derived DC change their phenotype and function following incubation with liposomes containing PS [27].

The serum β_2-glycoprotein I (β_2-GPI) is an aminophospholipid ligand that recognizes PS on the surface of apoptotic cells [28]. Opsonization of dying cells with β_2-GPI facilitates DC uptake of apoptotic cells and enhances presentation of MHC class-II-restricted apoptotic cell-derived peptides by DC to $CD4^+$ T cells [29].

Cells undergoing apoptosis are under considerable oxidative stress and generate reactive oxygen species that oxidize aminophospholipids (including PS) exposed on the cell surface. These oxidized lipids are structurally analogous to moieties detected on the oxidized low-density lipoprotein particle (Ox-LDL), which are recog-

nized by SR-A, SR-B1, CD36, CD68 and lectin-like O-LDL-R1 (LOX-1 or SR-E). It is believed that these phagocyte receptors may also bind oxidized aminophospholipids on the surface of apoptotic cells (Fig. 29.1). In fact, apoptotic cell uptake by macrophages can be inhibited *in vitro* by blocking the oxidized phospholipids present on the surface of apoptotic by means of specific monoclonal antibodies (mAb) [30]. The role of receptors for oxidized lipids in apoptotic cell recognition by DC is still unknown.

29.3.2
Thrombospondin-1 (TSP-1), CD36 and the Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$

TSP-1 is a platelet-derived multifunctional trimeric glycoprotein that interacts with cell surfaces and the extracellular matrix. The surface of apoptotic cells exposes anionic sites that allow deposition of TSP-1. By this mechanism, TSP-1 functions as a molecular bridge linking apoptotic cells with phagocytes. TSP-1 is recognized by the integrins $\alpha_v\beta_3$ (vitronectin receptor) and $\alpha_v\beta_5$ and the SR CD36 on the phagocyte surface. The $\alpha_v\beta_3$ integrin and CD36 recognize different regions of the TSP-1 trimer and only the $\alpha_v\beta_3$-TSP-1 interaction is blocked by Arg-Gly-Glu (RGD)-bearing peptides [31]. CD36 plays an amplifying role in the $\alpha_v\beta_3$-TSP-1 recognition, increasing the ability of phagocytes to internalize apoptotic cells [32, 33]. Human immature DC express $\alpha_v\beta_3$ [34] and $\alpha_v\beta_5$ [35] that cooperate with CD36 during recognition of TSP-1 on the surface of apoptotic cells. Human monocyte-derived DC downregulate surface expression of $\alpha_v\beta_5$ and CD36 following maturation *in vitro*, a fact that correlates with the reduced capability of mature DC for phagocytosis of apoptotic cells [35].

In mice, blockade of $\alpha_v\beta_3$ by means of mAb decreased the uptake of apoptotic cells by splenic DC *in vitro* [14] and only splenic CD8α^+ DC express high levels of CD36 on their surface [36, 37]. Interestingly, the subpopulation of CD8α^+ DC are the primary APC involved in crosspresentation of extracellular Ag via MHC class-I molecules to CD8$^+$ T lymphocytes [38]. In humans, it has been suggested that crosspresentation of apoptotic cell-associated Ag depends on the uptake of apoptotic cell fragments by DC via CD36 and $\alpha_v\beta_3/\beta_5$ integrins [34, 35]. However, splenic CD8α^+ DC from mice lacking CD36 or β_3/β_5 integrins crosspresent extracellular self- or foreign-Ag (associated with apoptotic or viable cells) as efficiently as their wild type counterparts [36, 37]. These results indicate that CD8α^+ DC must employ other receptors for internalization of extracellular Ag to be processed for crosspresentation. Alternatively, receptors required for apoptotic cell-clearance may operate at a high level of redundancy and other DC surface molecules may compensate for the lack of CD36 or β_3/β_5 integrins.

29.3.3
Complement Factors and Complement Receptors (CR)

The presence of heat-labile serum factors increases the uptake of apoptotic cells by phagocytes [39]. These factors have been identified as components of the comple-

ment system [40]. There is evidence that the surface of apoptotic cells can activate the complement cascade via the classical and the alternative pathways [40]. C1q, the first component of the classical pathway, binds to the surface of apoptotic cells by different mechanisms. Lysophosphatidylcholine exposed on the surface of apoptotic cells can be recognized by natural IgM antibodies [41, 42]. Binding of C1q to IgM attached to apoptotic cells initiates complement activation via the classical pathway, generating C3-derived opsonins that are recognized by phagocytes through CR.

The globular head of C1q may bind directly (without intermediate IgM) to poorly-characterized ligands that cluster on the surface membrane of apoptotic cell blebs [43, 44], a phenomenon that may trigger complement activation [45]. Alternatively, the collagen-like tail of C1q can bind calreticulin (also known as cC1qR) attached to the endocytic receptor CD91 (α-2-macroglobulin receptor) on the phagocyte surface [46] (Fig. 29.1B). By means of these mechanisms, C1q increases uptake of apoptotic cells by immature DC [47]. Interestingly, immature DC synthesize higher amounts of C1q than macrophages [48].

Other molecules in the plasma structurally related to C1q are the collectins, which include mannose-binding lectin (MBL) and lung surfactant protein A (SP-A) and D (SP-D). Collectins are characterized by their amino-terminal collagen-like region followed by a globular head with C-type lectin binding properties [49]. MBL and SP-A participate in uptake of apoptotic cells by attaching their globular domains directly to the surface of apoptotic cells. On the other end of the molecule, their collagen-like tails bind to the calreticulin/CD91 receptor on the surface of the phagocyte [50–53]. A recent report indicates that MBL enhances uptake of apoptotic cell by immature DC [47].

Mevorach et al. [40] have shown that externalization of PS by cells in early apoptosis was responsible in part for complement activation and iC3b deposition on the surface of apoptotic cells. In support of a direct role for PS in complement activation, PS-bearing micelles and cardiolipin (an anionic phospholipid similar to PS) were shown to activate complement [53]. Human monocyte-derived DC and mouse splenic DC employ the iC3b receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) to uptake iC3b-opsonized apoptotic cells *in vitro* [14, 54]. *In vivo*, splenic DC of mice made hypocomplementemic after injection of cobra venom factor (CVF) significantly decreased their ability to internalize circulating apoptotic cells [14]. CVF is a C3-like polypeptide that functions as an inactivation-resistance C3 convertase that, in mice, results in a severe inactivation of C3 (and secondary C5) without affecting factors like C1q that precedes C3 in the complement cascade. Therefore, complement factors derived from C3 or later in the cascade are of key importance for uptake of apoptotic cells by DC *in vivo* [14].

In physiological conditions, complement activation on the surface of apoptotic cells does not cause cell lysis, leakage of toxic mediators into the extracellular milieu or inflammation. One possible explanation for this paradoxical effect is that apoptotic cells control the generation of complement factors beyond iC3b by means of complement regulatory molecules. These regulatory factors prevent assembly of the C5b-C9 membrane attack complex (MAC) on the plasma membrane

of the apoptotic cell. One of these molecules is Factor H, a complement regulatory protein that accelerates the decay of the C3 and C5 convertases. Interestingly, the pentraxin C-reactive protein (CRP) binds to apoptotic cells and recruits Factor H on the surface of apoptotic cells, accelerating the dissociation of the C3 and C5 convertases and therefore decreasing generation of MAC [55]. A rapid clearance of apoptotic cells may also contribute to prevent generation of MAC on apoptotic cells [53]. The lack of an inflammatory reaction is due to the fact that binding of apoptotic cells via C3bi downregulates production of pro-inflammatory mediators by DC and macrophages [14].

29.3.4 Pentraxins

Pentraxins are acute-phase proteins secreted in response to inflammation. The name of this family of molecules is derived from the characteristic cyclic pentameric structure. It has been demonstrated that the pentraxins C reactive protein (CRP), serum amyloid P (SAP) and pentraxin 3 (PTX3) bind apoptotic cells *in vitro* [55–58]. Besides their binding to anionic phospholipids, CRP recognizes small nuclear ribonucleoproteins and SAP binds to chromatin/nucleolar components [57]. These intracellular molecules are mobilized to the cell surface during apoptosis and become accessible to pentraxins. The ligand(s) recognized by PTX3 on the apoptotic cell membrane is still unknown.

In the absence of complement, opsonization with SAP or CRP increases uptake of apoptotic cells by macrophages, indicating the existence of specific receptors for pentraxins [59]. It has been shown that pentraxins may also bind Fcγ receptors [60]. Unlike the pro-phagocytic effect of SAP and CRP, it has been shown that binding of PTX3 to apoptotic cells masks eat me signals required for uptake of apoptotic cells by immature DC [57].

29.3.5 Milk-fat Globule Protein Epidermal Growth Factor 8 (MFG-E8)/lactadherin

MFG-E8/lactadherin was originally identified on the surface of epithelial mammary cells and it is believed to participate in the apoptosis associated with mammary gland involution. MFG-E8 is secreted in two molecular variants, each containing two factor VIII-homologous regions and two epidermal growth factor (EGF) domains. MFG-E8 binds selectively to anionic phosholipids (PS and PE) by means of the VIII-homologous region [61]. The EGF domain includes an RDG motif that is recognized by the $\alpha_v\beta_3$ integrin [61]. Thus, MFG-E8 functions as a bridge between externalized PS on the surface of apoptotic cells and the $\alpha_v\beta_3$ integrin expressed by the phagocytes. Mouse immature DC and LC synthesize MFG-E8, and DC from MFG-E8-deficient mice are decreased drastically in their capability to internalize apoptotic cells [62]. In agreement with the fact that mature DC exhibit a limited ability for clearance of apoptotic cells, expression of MFG-E8 decreases in DC induced to mature by LPS [62]. Molecules like MFG-E8 and C1q, which are syn-

thesized by immature DC, may be critical for the uptake of apoptotic cells in those tissues where serum-derived factors may have limited access [63].

29.3.6
Other Apoptotic Cell Recognition Signals

A plethora of other, not so well characterized, ligand–receptor interactions has been observed to function in recognition and uptake of apoptotic cells. Most of these receptors have been studied in macrophages and information regarding their possible role in uptake of apoptotic cells by DC is limited or still unknown.

Apoptotic cells change the composition of sugars (*N*-acetylglucosamine, *N*-acetylgalactosamine) on the cell membrane. These modified carbohydrates can be recognized specifically by lectins expressed by phagocytes [64]. An asialoglycoprotein receptor identified on macrophages [65] and the lectin DEC-205 (CD205) expressed by $CD8\alpha^+$ DC, LC and thymic epithelial cells are good candidate receptors for apoptotic cell uptake via lectin–sugar recognition. DEC-205 is a receptor for endocytosis and delivery of Ag via DEC-205 to DC facilitates the recycling of the Ag through endosomes/lysosomes and enhances Ag presentation in the context of MHC class-II molecules [66]. Based on the fact that blockade of DEC-205 reduces uptake of apoptotic thymocytes by thymic epithelial cells *in vitro* [67], it is tempting to postulate that DEC-205 may participate in engulfment of apoptotic cells by $CD8\alpha^+$ DC. However, Iyoda and colleagues [26] have demonstrated that DC from DEC-205 deficient mice are still able to uptake apoptotic splenocytes. A possible explanation for this result is that DC from DEC-205 KO mice express other receptors for clearance of apoptotic cells that may compensate the lack of DEC-205.

The intracytoplasmic domain of the receptor Mer, a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family, has been shown to be critical for uptake of apoptotic cells in macrophages [68]. On the phagocyte surface Mer binds the soluble ligand Gas6, a product of the growth arrest-specific gene 6 that recognizes PS on the surface of apoptotic cells [69, 70]. By mediating the binding between Mer and PS, Gas6 functions as a molecular bridge between apoptotic cells and macrophages. Although murine BM-derived and splenic DC express Mer on their cell surface, DC from Mer-deficient mice engulf apoptotic cell as efficiently as DC of wild type controls [71].

The macrophage marker CD14 binds a structurally modified form of ICAM-3 that is present on the surface of apoptotic cells [72, 73]. The authors suggest that during apoptosis, ICAM-3 exposes/generates a new epitope that binds to CD14 and promotes apoptotic cell engulfment by macrophages [72, 73]. Apoptotic cell-clearance by DC is believed to be CD14-independent since human and murine DC do not express (or exhibit very low levels) of CD14.

Phagocytes can discriminate between apoptotic and viable cells by delivering detachment signals to living cells via homophilic interactions of CD31, a marker expressed by DC [74, 75]. By a poorly understood mechanism, apoptotic cells can switch the function of surface CD31 from repulsive to adhesive promoting tethering of dying cells to phagocytes [74].

29.4
Redundant Receptors and Backup Mechanisms for Apoptotic Cell Clearance

The numerous receptor–ligand interactions that participate in recognition and internalization of apoptotic cells by professional or semi-professional phagocytes may explain why, in normal conditions, the uptake of apoptotic cells is so efficient. The redundancy of receptors and backup mechanisms for apoptotic cell clearance is also an indirect indicator that removal of apoptotic cells must be extremely important to preserve the steady-state condition that characterizes healthy organisms [31]. In some cases, different receptors (i.e. CD36 and the $\alpha_v\beta_3$ integrin) cooperate mutually to increase the efficiency of apoptotic cell uptake [32, 33]. Receptors like Mer may be critical for some phagocytes (e.g. macrophages) [68] and play a redundant role or no function for other cell types (e.g. immature DC) [71]. Different ligand–receptor interactions may be required to recognize molecules expressed on the surface of apoptotic cells at different stages of apoptosis. Receptors that bind to PS may be important at early stages of apoptosis, when anionic phospholipids are exposed on the surface of apoptotic cells. At later stages of apoptosis, when intracellular molecules are exposed de novo on the surface of apoptotic cells, the binding of soluble pentraxins may play an important role in apoptotic cell recognition and clearance by phagocytes.

29.5
Regulatory Effects of Early Apoptotic Cells on Dendritic Cells

The normal turnover of billions of tissue cells that die by apoptosis every day in our body is not accompanied by inflammation. Likewise, conditions causing apoptotic cell death (e.g. moderate UV-B irradiation, certain viral infection, tumor growth, etc) are characterized by lack of inflammation and suppression of T cell-mediated immunity. It was originally thought that the high efficiency of the clearance of apoptotic cells was the simple reason by which apoptotic cells failed to provide the danger signals required to initiate inflammation. Voll and colleagues [76] were the first to demonstrate that apoptotic cells suppress the inflammatory response through an active mechanism that includes delivery of inhibitory signals to the phagocytes of the immune system. More recently, several laboratories have demonstrated that internalization of cells in early apoptosis by immature DC is not accompanied by DC activation in humans and rodents [77, 78]. Internalization of apoptotic cells by immature DC is not an immunologically null event. Immature DC that have internalized cells in early apoptosis fail to upregulate the expression of MHC-II, CD80, CD86, CD40 and CD83 (in humans) even after subsequent stimulation with different DC-maturation factors including LPS, CD40 ligation, TNF-α and monocyte-conditioned medium [54, 78–80]. This inhibitory effect is not merely the consequence of phagocytosis of particulate Ag, since immature DC do not exhibit defects in phenotype or maturation driven by LPS after ingestion of control latex beads of similar size than apoptotic cell fragments [78]. Following uptake of

apoptotic cells, DC decrease their capacity to stimulate Ag-specific TCRtg T cells and allogeneic T cells [78, 79]. DC exposed to apoptotic cells exhibit a limited capacity for presentation of antigenic peptides to T-cell clones that have less stringent requirements for co-stimulation than primary T lymphocytes [80]. Collectively, these results suggest that the defect of DC exposed to apoptotic cells is probably at the level of expression of MHC-peptide complexes and co-stimulatory molecules on the cell surface, rather than a deficiency in the Ag-processing capacity of DC [80]. By contrast, immature DC exposed to necrotic cells or their supernatants, increase expression of MHC and co-stimulatory molecules and exhibit an augmented capacity to stimulate $CD4^+$ or $CD8^+$ T cells [13].

As described initially in macrophages [76, 81, 82], DC that have interacted with and/or internalized cells in early apoptosis secrete significantly lower levels of pro-inflammatory mediators, including IL-1α, IL-1β, IL-6, IL-12p70 and TNF-α [14, 80].

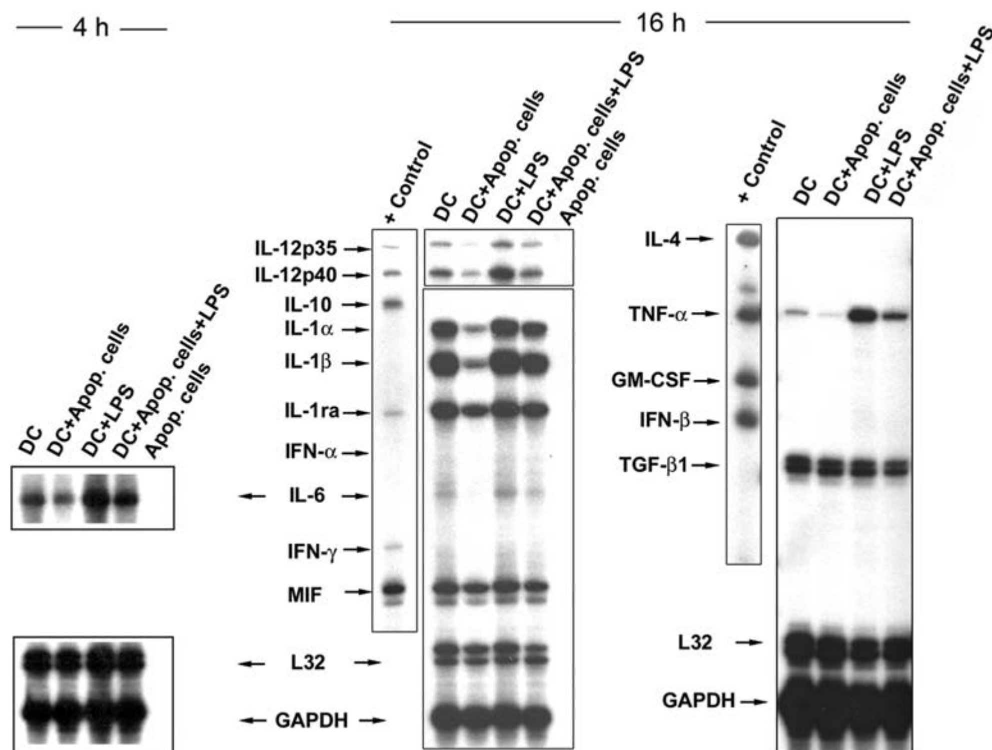

Fig. 29.2 Effect of apoptotic cells on DC cytokine gene mRNA transcription or stabilization. Comparative RNAse protection assay analysis of cytokine mRNA transcribed by DC following phagocytosis of apoptotic splenocytes. mRNA was isolated from immunomagnetic bead-sorted BM-derived DC after co-incubation with apoptotic cells for 4 or 16 h, in the absence or presence of 200 μg ml^{-1} LPS. For most cytokines, the changes in mRNA levels were evident after 16 h with the exception of IL-6. Reproduced with permission from *Blood* **2003**. 101: 611–620 (A.E. Morelli et al.).

Other groups have reported that DC exposed to apoptotic cells may increase secretion of IL-6, IL-12p40 and TNF-α [78, 79]. These contrasting results may be due to different experimental conditions or exposure of DC to cells in later stages of apoptosis. The inhibitory effect of apoptotic cells on secretion of pro-inflammatory cytokines by DC was maintained even in the presence of LPS [14]. Interestingly, internalization of apoptotic cells did not interfere with secretion of immunosuppressive cytokines like TGF-β1 by murine DC [14] and increased release of IL-10 by human DC [80]. At least part of the inhibitory effect of apoptotic cells on cytokine production by DC is at the level of cytokine mRNA transcription or stabilization (Fig. 29.2) [14].

It has been shown that macrophages with internalized apoptotic cells inhibit release of pro-inflammatory mediators and augment secretion of TGF-β1, prostaglandin E2 (PGE2) and platelet-activating factor [82]. Release of soluble TGF-β1, PGE2 and platelet-activating factor inhibited by means of a paracrine mechanism secretion of pro-inflammatory cytokines by bystander macrophages that were not exposed to apoptotic cells [82]. By contrast, modulation of the DC function by apoptotic cells appears to be exclusively a cell contact-dependent process. Stuart and colleagues [78] have demonstrated that the inhibitory effects of apoptotic cells were restricted to those DC that have engulfed apoptotic cells, while bystander DC remained unaffected. Unlike macrophages, the inhibitory effects of apoptotic cells on DC were not mediated by soluble cytokines like TGF-β1 or IL-10 [78, 80].

29.6 Molecular Mechanisms of the Interaction between Dendritic Cells and Apoptotic Cells

As were originally demonstrated in macrophages [76], apoptotic cells signal DC through some of the surface receptors that participate in recognition/uptake of apoptotic cells. Urban et al. [80] have demonstrated that human immature DC exposed to mAb against CD36 or the α_v-integrin chain (CD51) evoked a minimal allogeneic T-cell response and failed to mature in response to LPS, CD40 ligation, TNF-α and monocyte-conditioned medium. Interestingly, exposure of DC to anti-CD36 mAb also increased secretion of IL-10 and inhibited release of IL-12p70 in response to LPS [80].

Apoptotic cells can interact with DC via CR. In humans, DC exposed to iC3b-opsonized apoptotic cells remained immature, did not secrete IL-12 and failed to upregulate surface HLA-DR and CD86 after stimulation with anti-CD40 mAb or LPS [54]. In mice, co-incubation of BM-derived DC with erythrocytes bearing IgM and mouse iC3b on their surface inhibited DC secretion of IL-1α, IL-1β, IL-6, IL-12p70 and TNF-α even in the presence of LPS, but it did not affect release of TGF-β1 [14]. The effect of iC3b on DC cytokines was at least in part mediated via CD11b/CD18 (CR3), since a similar modulation of cytokines can be obtained following exposure of DC to polystyrene beads coated with mAb that binds to CD11b or CD18 [14]. These results agree with previous reports that showed that binding of iC3b to CR3

inhibits IL-12p70 secretion by macrophages [83, 84] and that local deposition of iC3b is critical for UV-B-mediated skin immunosuppression, an effect that requires interaction of iC3b with a subset of dermal macrophages [85, 86]. The internalization of C1q-opsonized apoptotic cells, but not nonopsonized apoptotic cells, by DC stimulated production of IL-6, IL-10 and TNF-α without increasing IL-12p70 secretion [87].

CD47 (also known as integrin-associated protein) is a molecule present on the plasma membrane that is physically and functionally coupled to the $\alpha_v\beta_3$ integrin (Fig. 29.1). This multispan transmembrane protein is expressed by immature DC and recognizes TSP as its ligand. Although blockade of CD47 does not affect the ability of human DC to engulf apoptotic cells, there is evidence that apoptotic cells might modulate the function of DC through CD47. Engagement of CD47 on the surface of human monocyte-derived immature DC, by means of mAb or a TSP-derived synthetic peptide, inhibited DC maturation and secretion of IL-6, IL-12p70 and TNF-α in response to bacterial stimulation [88].

Fadok and colleagues have demonstrated that, in macrophage cell lines, binding of the stereospecific receptor for PS decreases secretion of TFN-α and augments release TGF-β1 in response to LPS [23].

29.7
Dendritic Cells, Apoptotic Cells and Peripheral Tolerance

DC play a dual role in the immune system, they initiate T-cell immunity and participate in central and peripheral T-cell tolerance. In the thymus, DC delete those thymocytes with high affinity for self MHC-peptide complexes. However, central deletion of self-reactive thymocytes is incomplete and not all self-Ag have permanent access to thymic APC because they are expressed later in life (e.g. puberty), are synthesized transiently (e.g. pregnancy) or remain sequestered in peripheral tissues (e.g. myelin basic protein). Self-reactive T cells that escape thymic deletion have access to peripheral tissues and may eventually trigger autoimmunity. An efficient mechanism of peripheral T-cell tolerance must constantly monitor and inactivate/delete any possible self-reactive T cells and/or induce/amplify T cells with regulatory function [regulatory T (T_{reg}) cells].

The current DC-based models of peripheral T-cell tolerance propose that immature/semi-mature DC migrate constitutively from peripheral tissues to secondary lymphoid organs as “Ag transporting cells” [89–91]. These migratory DC, expressing self-peptides-MHC complexes and with weak T-cell stimulatory function, would induce anergy and/or apoptosis of autoreactive T cells or generation of self-Ag-specific T_{reg} cells [89, 90]. Experiments in mice expressing transgenic model Ag controlled by tissue-specific promoters have demonstrated that, in the steady state, constitutively migratory DC transport and process tissue-specific Ag from periphery to lymph nodes and spleen. These DC silence, instead of stimulate, autoreactive $CD4^+$ or $CD8^+$ T cells [92, 93]. In humans, repetitive stimulation of naïve $CD4^+$ T cells by allogeneic immature DC reportedly generates T_{reg} cells [95]. Administra-

tion of immature DC pulsed with an MHC class-I restricted influenza-derived peptide leads to specific inhibition of Ag-specific $CD8^+$ T cells [96, 97].

A critical point of the current model of peripheral T-cell tolerance is that migratory DC transport self-Ag from the periphery to secondary lymphoid organs. Apoptotic cells that result from the normal cell turnover of peripheral tissues are a rich source of self-Ag and are internalized efficiently by immature DC of peripheral tissues. Interestingly, Ip and colleagues [98] have reported that following ingestion of apoptotic cells, DC decreased expression of the chemokine receptor CCR5 and increased the levels of CCR7. This result indicates that DC with internalized apoptotic cells, although remaining immature, acquire the ability to home to draining lymph nodes. Huang et al. [99] reported that intestinal DC with intracellular apoptotic cell fragments (derived from intestinal epithelial cells) traffic to mesenteric lymph nodes. This observation suggests that internalization of apoptotic cells by DC in peripheral tissues followed by transport and presentation of self-peptides to naïve T cells in secondary lymphoid organs plays a role in induction/maintenance of peripheral T-cell tolerance [99]. Experiments in gnotobiotic rats under germ-free conditions indicate that migration of intestinal DC with phagocytosed apoptotic cells towards the mesenteric lymph nodes is a constitutive phenomenon, independent of the presence of DC-maturation stimuli derived from the intestinal bacterial flora [99]. The presence of DC with engulfed apoptotic bodies or with intracellular inclusions derived from neighboring parenchymal cells has been reported in other peripheral tissues. LC with internalized apoptotic keratinocytes derived from epithelial cells have been detected in the vaginal epithelium of mice [7]. In the skin, epidermal LC internalize melanosomes released by surrounding melanocytes and skin-draining lymph nodes contain DC with phagocytosed melanosomes [100]. In mice, Scheinecker et al. [101] have detected DC with intracytoplasmic fragments containing proton pump H^+/K^+-ATPase (a self-Ag expressed by parietal cells) close to the gastric epithelium and in T-cell areas of stomach-draining lymph nodes. These DC efficiently presented an H^+/K^+-ATPase-derived peptide to specific TCR tg T cells [101]. Blood-borne apoptotic/dying cells are also captured efficiently from circulation by splenic DC (Fig. 29.3) [14, 26]. Iyoda et al. [26] have shown that circulating leukocytes subjected to apoptosis by osmotic shock are internalized *in vivo* exclusively by splenic $CD8\alpha^+$ DC within the T cell-areas. By contrast, our group [14] has shown that blood-borne (UV-B-induced) allogeneic apoptotic leukocytes are captured initially by splenic $CD8\alpha^-$ DC of the marginal zone. These marginal zone DC might later acquire $CD8\alpha$ and mobilize to T-cell areas, or alternatively, transfer the apoptotic cell fragments to $CD8\alpha^+$ DC, as suggested by others [102]. However, it is likely that those dying blood-borne cells still able to traffic to T cells areas of the spleen before becoming apoptotic are phagocytosed directly by $CD8\alpha^+$ DC and those circulating apoptotic cells unable to migrate to T-cell areas are captured easily by marginal zone $CD8\alpha^-$ DC.

Internalization of apoptotic cells by splenic DC is associated with the presentation of apoptotic cell-derived peptides in MHC class-I and -II molecules to $CD8^+$ and $CD4^+$ T cells, respectively [26]. Liu et al. [103] have shown that, in the steady state, presentation of apoptotic cell-derived peptides by immature/semi-mature

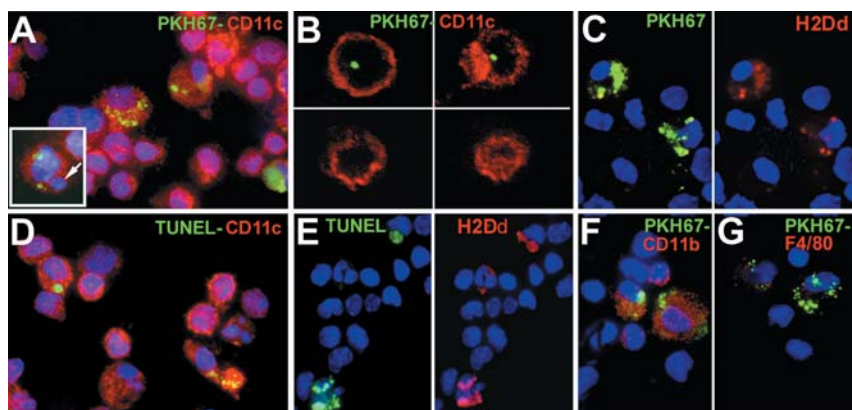

Fig. 29.3 Entrapment of apoptotic cells by splenic DC *in vivo*. Internalization of apoptotic cells by splenic DC was analyzed in cytospins of immunobead-sorted DC 1 h after injection of PKH67-labeled (green) apoptotic (BALB/c) splenocytes in (B10) mie. (A) CD11c$^+$ DC with apoptotic cell fragments (green) and with DAPI$^+$ intracytoplasmic inclusions, -likely DNA from ingested apoptotic cells (in blue indicated by arrow in inset). (B) Serial sections analyzed by confocal microscopy confirmed the intracellular localization of PKH67-labeled fragments in splenic CD11c$^+$ DC. (C) The donor origin (BALB/c) of the intracytoplasmic inclusions in (B10) DC was confirmed by H2D^d expression (in red) in PKH67-labeled (green) fragments. (D & E) FITC-TUNEL staining in combination with Cy3-anti-CD11c or Cy3-anti-H2D^d confirmed the presence of donor (BALB/c)-derived apoptotic cells within (B10) DC. (F & G) One h after i.v. injection of apoptotic cells, DC that internalized apoptotic cells expressed CD11b^{hi} and F4/80$^{lo/-}$. Nuclei were counterstained with DAPI (1000x). Reproduced with permission from *Blood* **2003**. 101: 611–620 (A.E. Morelli et al.).

DC in secondary lymphoid organs leads to peripheral CD8$^+$ T-cell tolerance. Following i.v. injection of apoptotic/dying leukocytes loaded with the model Ag OVA, splenic DC internalized the circulating apoptotic cells and presented an OVA-derived peptide to TCR tg CD8$^+$ cells [103]. Since internalization of apoptotic cells did not lead to APC maturation, DC expressing inadequate levels of co-stimulatory molecules induced abortive proliferation of OVA-specific CD8$^+$ T cells followed by deletion and CD8$^+$ T-cell tolerance to antigenic challenge [103].

29.8
The Potential Therapeutical Use of Apoptotic Cells for Peripheral Tolerance

Based on the current model of peripheral T-cell tolerance, specific targeting of Ag to immature/semi-mature DC in secondary lymphoid organs *in situ* (in the steady state) is a novel approach to induce T-cell tolerance for treatment of transplant rejection or autoimmune disorders [91]. To adopt this principle to achieve T-cell tolerance, the method employed to deliver the Ag to the host DC *in situ* should avoid the activation of the acceptor DC, while maintaining their intrinsic capacity to

present the Ag in a tolerogenic fashion [91, 104]. Importantly, this new approach would prevent the spontaneous DC activation induced by manipulation of DC generated *in vitro*, which has been a drawback in the current DC-based therapies for tolerance induction [91]. Interestingly, it has been demonstrated in mice that specific targeting of model Ag to immature/semi-mature DC in lymphoid tissues by delivering the HEL_{46-61} peptide attached to an anti-DEC-205 mAb (DEC-205 is expressed by DC in secondary lymphoid organs) induced a sustained reduction of the T-cell response to HEL_{46-61} challenge [105, 106]. Ferguson et al. [107] have demonstrated that i.v. administration of trinitrophenyl (TNP)-coupled splenocytes that become apoptotic (via a Fas-FasL dependent pathway) soon after injection induce hapten-specific T-cell unresponsiveness in a model of contact hypersensitivity in mice. The phenomenon was mediated by host splenic $CD8\alpha^+$ DC and the inhibitory effect on contact hypersensitivity could be adoptively transferred from unresponsive animals to untreated hosts, suggesting the generation of T_{reg} cells [107].

Based on the current model of peripheral T-cell tolerance, administration of donor cells (expressing MHC class-I and –II) in early apoptosis to deliver alloAg to host semi-mature DC in secondary lymphoid tissues (without disrupting the steady state) may be an effective way to induce donor-specific tolerance for transplantation. Early apoptotic cells have the following advantages for specific targeting of alloAg to DC *in vivo* compared to other systems: (i) apoptotic cells are a rich source of alloAg; (ii) unlike soluble MHC, MHC peptides, chimeric MHC molecules and Ag tagged to mAb, early apoptotic cells are easy to prepared in the laboratory; (iii) i.v. administration of allogeneic apoptotic cells is relatively safe; (iv) once injected i.v., blood-borne apoptotic cells are captured efficiently by splenic DC [14, 26]; (v) early apoptotic cells deliver a potent immunosuppressive signal to DC [14, 54, 78]; and (vi) DC present apoptotic cell-derived allopeptides [108].

In this regard, we have demonstrated that i.v. injection of donor apoptotic splenocytes (in early apoptosis) 7 days before transplant surgery prolongs survival of cardiac allografts in the absence of immunosuppression in a murine experimental model [109]. The effect was donor-specific and depended on injection of donor cells in early apoptosis, since administration of donor necrotic cells did not affect graft survival. In this model, splenic DC rapidly ingested the i.v. injected apoptotic cells, processed apoptotic cell-derived peptides into MHC molecules and mobilized to T-cell areas of the splenic follicle. Administration of donor apoptotic leukocytes before transplant surgery drastically reduces the systemic and local anti-donor response. The effect requires the interaction of circulating apoptotic donor cells with recipient splenic $CD8\alpha^+$ DC [109]. In agreement with our results, it has been shown that human immature DC loaded with allogeneic apoptotic cells induce cross-tolerance to allospecific T cells *in vitro* [108]. Using a more indirect approach, Xu et al. [110] have recently shown in mice that intra-portal administration of recipient immature BM-derived DC loaded *in vitro* with donor apoptotic splenocytes prolongs survival of cardiac allografts in the absence of pharmacological immmunosuppression.

In a different transplantation model, de Carvalho Bittencourt and colleagues [111] have shown that i.v. injection of donor apoptotic splenocytes enhances BM

engraftment in mice. Interestingly in this study, the therapeutic effect of i.v. administration of apoptotic leukocytes was nonspecific and independent of the origin of the apoptotic bodies (donor, recipient, third party or xenogeneic) [111]. Of note, in this study apoptotic cells were administered simultaneously with the BM inoculum [111]. Therefore, it is possible that the interaction of host DC with a dose of self, third party or xenogeneic apoptotic cells may have exerted a bystander inhibitory effect on DC while presenting donor BM-derived alloAg to anti-donor T cells.

In humans, there is evidence that photopheresis exerts beneficial prophylactic and therapeutic effects on survival of cardiac allografts [112]. During this process, extracorporeal UV-irradiation of mononuclear cells triggers their apoptosis after re-infusion into the patient. Mechanisms underlying the inhibitory effects of apoptotic cells in photopheresis-treated patients have yet to be clarified, but modulation of the recipient DC function by circulating apoptotic cells may probably be involved in the therapeutic effect.

29.9 Pathogens and Apoptotic Cell-like Mimicry

Clearance of apoptotic cells by phagocytes is a system highly conserved through evolution, extremely efficient and designed to prevent tissue damage and development of immunity. Therefore it is not strange that certain pathogens have evolved to target this mechanism to suppress immunity and to establish infection. The malaria parasite *Plasmodium falciparum* employs some of the mechanisms used for clearance of apoptotic cells to evade the host immune response. DC exposed to malaria-infected red blood cells failed to mature in response to LPS and to induce primary and secondary Ag-specific T-cell responses [113]. The inhibitory effect of parasite-infected erythrocytes on DC was mediated by direct binding of the erythrocytes to CD36 and indirectly, through attachment of the bridging molecule TSP-1, to CD36 and $\alpha_V\beta_3/\beta_5$ on the DC surface [80]. By this mechanism malaria-infected erythrocytes prevented DC maturation and shifted secretion of cytokines from IL-12p70 to IL-10 [80]. Thus, parasite-infected erythrocytes downregulated the function of DC as occurs during interaction with apoptotic cells, a phenomenon that may explain the impairment of T-cell immunity in patients with malaria. *Trypanosoma cruzi* is another example of a parasite that takes advantage of the mechanism of apoptotic cell clearance for its survival. In a model of Chagas disease, phagocytosis of apoptotic cells by macrophages via the $\alpha_v\beta_3$ integrin induces secretion of TGF-β1 and prostaglandin E_2 that promote growth of *T. cruzi* [114].

P. Vanlandschoot and G. Leroux-Roels [115] have postulated the hypothesis that the hepatitis B virus (HBV) may affect the function of DC and alter the Th1/Th2 balance in detriment of secretion of IL-12 and IFN-γ by mimicking mechanisms employed by apoptotic cells to maintain self-tolerance. HBV-infected hepatocytes release noninfectious subviral particles into circulation, known as hepatitis surface Ag (HBsAg). It is unclear, how the massive number of HBsAg particles produced during hepatitis B participates in establishment and maintenance of HBV infec-

tion. Interestingly, blood-borne HBsAg display phospholipids and oxidized phospholipids on their surface, similar to those present on the surface of apoptotic cells [115]. These HBsAg phospholipids bind soluble β2-GPI and CD14 (and probably other receptors for phospholipids) as demonstrated in apoptotic cells. Alternatively, circulating complexes of HBsAg and IgM/IgG may induce deposition of complement factors that opsonize HBsAg particles facilitating their interaction with DC [115]. Yeast-expressed HBsAg behave as apoptotic cell-like particles inhibiting LPS-induced secretion of pro-inflammatory cytokines and augmenting IL-10 produced by monocytes [116]. The possible apoptotic cell-like interaction of HBsAg particles with DC and its impact on DC function are still unknown.

29.10
Dead Cells and the Delicate Balance between Immunity and Tolerance

Dead cells exert different effects on DC. Several groups have demonstrated that exposure of DC to necrotic cells, but not apoptotic cells, induce APC maturation and generation of Ag-specific $CD4^+$ and $CD8^+$ T cells [13, 77]. Necrotic cell death, unlike apoptosis, has been traditionally associated with inflammation and initiation of adaptive immunity. Since the initial observation by Sauter et al. [13] demonstrating that filtered supernantants from necrotic cell lines induced DC maturation, the nature of soluble mediators released by necrotic cells and responsible for DC activation are beginning to be elucidated. Cells undergoing necrosis passively release uric acid, heat shock proteins (HSP) and high mobility group box 1 (HMGB1), all molecules with potent pro-inflammatory effects [117–119]. HSP are released from necrotic or stressed cells but are confined within apoptotic cells, unless the latter are disrupted or undergo secondary necrosis [117]. Once in the extracellular milieu, HSP bind to receptors on the surface of DC (e.g. CD91) and induce their activation [117]. HMGB1 is a chromatin-binding protein that binds the receptor for glycosylation end products (RAGE), activates the nuclear factor-κB (NF-κB) signaling pathways and induces DC maturation [120]. Interestingly, apoptotic cells do not release HMGB1, a phenomenon due to active de-acetylation of histones that result in tight binding of HMGB1 to the chromatin of apoptotic bodies.

The idea that DC distinguish between the two types of cell death and decide the outcome of adaptive immunity accordingly, with necrotic cells providing information for initiation of immunity and apoptotic cells for induction/maintenance of tolerance, is a generalization of a far more complex phenomenon. If DC-activating signals came exclusively from necrotic cells, all pathogens that induce apoptosis would escape immunity. Several studies have shown that DC loaded with apoptotic cells activate $CD4^+$ [9, 10] and cross-prime $CD8^+$ T cells efficiently [8–10]. Although, it may be argued that some of these studies may have employed a mixed population of apoptotic and necrotic cells or that the apoptotic cells have been stressed or infected with pathogens, there is evidence that, under certain conditions, apoptotic cells may initiate inflammation and/or adaptive immunity. In this

regard, the type of opsonin deposited on the surface of apoptotic cells may exert different effects on the function of the acceptor phagocytes. Endocytosis of IgG-opsonized apoptotic cells by macrophages via Fc receptors did not prevent LPS-induced secretion of pro-inflammatory cytokines, as occurs after internalization of control apoptotic cells [82]. In certain autoimmune disorders, the phagocyte receptors employed to internalize apoptotic cells may activate different downstream signaling pathways and perturb the anti-inflammatory effect of apoptotic cells. Patients with systemic lupus erythematosus (SLE) exhibit circulating anti-phospholipid auto-antibodies that recognize externalized phospholipids on the surface of apoptotic cells [121]. Internalization of these Ig-opsonized apoptotic cells can stimulate secretion, instead of suppression, of TNF-α by macrophages and other phagocytes [121].

Impairment or saturation of the mechanism of clearance of cells in early apoptosis may lead to accumulation of cells in late apoptosis (or secondary necrosis) with the consequent release of toxic mediators that may eventually induce DC activation. This phenomenon may be triggered by the simple increase of the ratio between apoptotic cells and DC. Incubation of DC with an excess of apoptotic cells was associated with DC maturation and efficient presentation to both MHC class I- and class-II restricted T cells [10]. The effect was apoptotic cell dose-dependent and accompanied by an autocrine/paracrine secretion of IL-1β and TNF-α [10]. Moreover, immunization with high numbers of apoptotic cells or with DC exposed to high numbers of apoptotic cells primed tumor-specific CTL and conferred protection against tumor challenge in mice [122]. Alternatively, accumulation of cells in late apoptosis may be caused by a deficiency in the mechanism of recognition/docking/internalization of apoptotic cells. In humans and mice, defective clearance of apoptotic cells is associated with development of an SLE-like autoimmune disorder. Apoptotic cells are thought to be a major source of auto-Ag in SLE in humans. Mice deficient in molecules involved in the uptake of apoptotic cells such as C1q, C4, IgM, SAP, and c-Mer develop systemic autoimmunity [123–128]. SLE in humans is associated with genetic deficiencies in the complement factors C1q, C1r, C1s, C4 and C2 [129], and phagocytes from patients with SLE exhibit impaired uptake of apoptotic cells [130, 131]. There is a hierarchical association between SLE susceptibility and severity of the disease according to the position of the complement factor in the activation pathway, with the highest incidence of the disease in patients with C1q deficiency [132].

Exposure of DC to APC-activating signals while ingesting apoptotic cells may also affect the outcome of the T-cell response. Several viruses induce apoptosis of their host cells and different types of virus-infected cells secrete IFN-α. DC that have internalized apoptotic fibroblasts and that otherwise would remain immature, become activated following incubation with IFN-α [77]. Therefore, pathogen-induced secretion of DC-activating cytokines (e.g. IL-1β, TNF-α, IFN-α) may help to explain those studies where DC primed specific T cells efficiently following uptake of apoptotic cells derived from monocytes infected by influenza A virus [8] or from macrophages infected by *Salmonella typhimurium* [9]. Alternatively, DC also

could receive maturation-signals via $CD4^+$ T cell-dependent CD40 stimulation, viral double stranded RNA, CpG DNA motifs, LPS from gram-negative bacteria, HSP from stressed apoptotic cells or HSP and HMGB1 from necrotic cells.

The intrinsic characteristics of the apoptotic cells may also influence the capability of the acceptor APC to process and present apoptotic cell-derived Ag and to shift the balance between immunity and tolerance [133]. Gao et al. [134] and Chen and colleagues [135] have shown that apoptotic leukocytes can release IL-10 and bio-active TGF-β1, two cytokines that exert immunoregulatory effects on DC. By contrast, heat stress can upregulate expression of molecules that induce DC activation, such as HSP72 and HSP60 on the surface of apoptotic cells [136]. Vaccination with DC pulsed with heat-stressed apoptotic tumor cells results in development of tumor-specific immunity in mice [136]. A study by Lauber and colleagues [137] showed that apoptotic cells release lysophosphatidylcholine, a lipid chemotactic for monocytes and macrophages. Lysophosphatidylcholine is produced by hydrolysis of membrane phosphatidylcholine by phospholipase A2 (PLA2) and calcium-independent PLA2 is activated by cleavage by caspase 3 during apoptosis. By binding the immunoregulatory receptor G2A, lysophosphatidylcholine can activate pro-inflammatory transcription factors like NF-κB and signaling through mitogen-activated protein kinase (MAPKs) pathways. Therefore, local release of lysophosphatidylcholine by apoptotic cells may eventually create a pro-inflammatory environment for macrophages and DC.

29.11 Concluding Remarks

During the last ten years, the better understanding of the mechanisms of interaction of professional APC with apoptotic cells has opened new possibilities to deliver exogenous Ag to DC for immunization or negative vaccination. Many variables including the intrinsic properties of apoptotic cells and their interactions with receptors on the surface of DC will have to be optimized in view of possible therapeutic uses of apoptotic cells in the future. These approaches may include administration of DC loaded with apoptotic cells for treatment of infectious diseases or malignant neoplasms, or alternatively, for induction of Ag-specific tolerance for prevention/treatment of autoimmune disorders or allograft rejection. Still from the grave, a dead cell has stories to tell.

Acknowledgements

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30
Pharmacologically Modified Dendritic Cells: A Route to Tolerance-associated Genes

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30.1
Dendritic Cells, Maturation and Tolerance

Just as the immune system has evolved to ensure wide-ranging efficacy in combating harmful agents, so it has also incorporated multiple layers of negative control to minimize self-harm, localizing and limiting the extent of productive responses, as well as preventing the initiation of inappropriate immunity. For example, although autoreactive T cells are deleted in the thymus during the establishment of central tolerance, this process is incomplete and peripheral tolerance mechanisms are required to control escaping self-reactive T cells [1], in addition to tempering T cells potentially reactive against harmless ubiquitous agents such as inhaled antigens and gut flora.

It has been demonstrated that dendritic cells (DCs) play a role in the induction and maintenance of peripheral T-cell tolerance by a variety of incompletely understood mechanisms, including deletion, induction of anergy or hyporesponsiveness and the generation of regulatory T cells, Tregs [2, 3]. $CD4^+CD25^+Foxp3^+$ T cells, “natural” regulators, although typically considered to arise in the thymus, do also appear to be generated extrathymically [4, 5], and while it is known that they require antigen stimulation and IL-2 to trigger their suppressive effects, the ensuing suppression of pathogenic T cells is mediated, at least in *in vitro* readouts, by an as yet elusive, antigen-nonspecific, cell-contact-dependent mechanism [6]. Tr1-like cells, “adaptive” regulators on the other hand, are differentiated extrathymically from $CD4^+CD25^-$ cells, typically in the presence of IL-10 [7], and are considered to represent a specialized activation state of conventional $CD4^+$ cells. Indeed T helper (Th) 1/Th2 polarized Tr1-like cells have been differentially generated following respiratory challenge [8, 9]. Tr1-like cells are thought to regulate T-cell responses in an antigen-specific, contact-dependent manner through the action of IL-10 and/or TGFβ [10]. The exact molecular signals conveyed by DCs leading to the generation of Tregs are currently unknown.

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DCs distribute throughout the periphery where they act as "sentinels" for both immunity and tolerance [2, 11]. While immature DCs exhibit immense capacity to continuously sample their antigenic micro-environment, these cells exhibit low surface MHC class II and co-stimulatory molecule expression and the sampled antigen is not displayed at the cell surface. In response to appropriate stimuli DCs mature, rapidly losing their capacity for antigen uptake and acquiring increased surface expression of MHC and co-stimulatory molecules. This maturation is accompanied by alterations in chemokine and chemokine receptor expression that facilitate exit from the inflammatory site and homing to the T-cell areas of the lymph node where they can now potently stimulate naïve T cells (Fig. 30.1) [11–15]. IL-6 generated by DCs in response to microbial stimuli causes $CD4^{+}CD25^{-}$ effector T cells to become refractory to suppression by $CD4^{+}CD25^{+}$ Tregs promoting T-cell immunity [16]. Surface MHC:peptide and co-stimulatory molecules provide what have been termed signals 1 and 2 that are necessary for effective T-cell priming, while a third signal is provided in the form of polarizing Th1 or Th2 cytokines and is determined by the nature of the activation stimulus [17, 18]. The capacity of DCs to activate T cells appears closely related to their level of maturation and this in turn is influenced by a variety of factors, including the nature of the stimulus, the relative responsiveness of the DC lineage or subtype and the cytokine micro-environment in which the stimulus is encountered (Fig. 30.1). In the absence of relevant pathogen derived stimuli, DCs require a maturation boost from Th cells to achieve priming of naïve $CD8^{+}$ cells and the development of effector cytotoxic T lymphocytes (CTLs), a process that has been termed "conditioning" or "licensing" of the DC [19–21]. Cross-presentation of self-antigen in the absence of licensing results in deletion of self-reactive CTLs and "cross-tolerance" [22–24]. In a similar manner, activation of DCs by inflammatory mediators in the absence of microbial stimulation is sufficient to generate DCs that are phenotypically mature with respect to upregulation of MHC II and co-stimulatory molecules, and which can support $CD4^{+}$ T-cell clonal expansion, but which fail to direct Th differentiation [25].

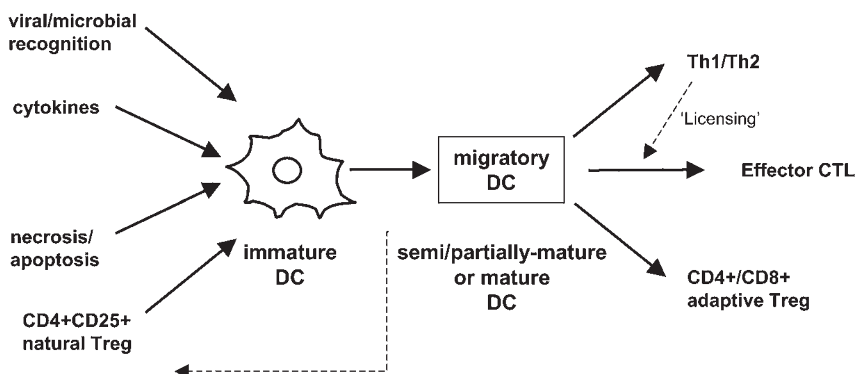


Fig. 30.1 The functional phenotype of dendritic cells is related to their maturation state, the level of which represents a balance of environmental influences and cellular responsiveness.

Regulating the state of DC maturity appears to be a key pivot point in the maintenance of an appropriate balance between immune activation and tolerance. The tolerogenic nature of antigen presentation by immature DCs has been illustrated by repetitive *in vitro* stimulation of naïve $CD4^+$ T cells, which caused the differentiation of Tr1-like cells [26, 27], while a single injection of influenza matrix peptide-pulsed immature DCs into human volunteers caused transient, antigen-specific inhibition of CTL responses and the induction of IL10-producing, contact-dependent, regulatory $CD8^+$ T cells [28, 29]. Using an inducible transgenic mouse model to avoid perturbations that could be associated with *ex vivo* manipulation and adoptive transfer of resting DCs, presentation of an LCMV-derived CTL epitope similarly resulted in antigen-specific $CD8^+$ tolerance, which could not be broken by subsequent infection with LCMV [30]. Conjugation of antigen or antigenic peptide to an antibody targeting the DC-specific lectin DEC-205 has been used to deliver antigen to immature DC *in vivo via* endocytosis, a process that in itself does not induce DC maturation. While the simultaneous delivery of a maturation stimulus generated immunity, delivery of the antigen-anti-DEC-205 species alone initiated a burst of T-cell proliferation followed by deletion and the induction of antigen-specific unresponsiveness [31, 32], and the induction of $CD25^+$ Treg cells [33].

It has been proposed that apoptotic cells can act as a reservoir of self-antigen for the maintenance of peripheral tolerance and that while circulating apoptotic particles can be captured and presented directly by lymph-node or spleen resident DCs in the steady state [34, 35], under non-inflammatory conditions self-antigens can also be captured by DCs in the periphery and transported to lymph nodes *via* the afferent lymph [36]. Uptake of apoptotic cells, in contrast to the uptake of necrotic cells, does not mediate maturation of DCs, but does result in a decreased production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-12p70 and TNFα [34, 37, 38]. Rather than becoming resident in the T-cell areas of the nodes themselves, it has been suggested that these migratory DCs may actually die rapidly and be processed by lymph-node resident DCs that then tolerize autoreactive $CD4^+$ and cross-tolerize autoreactive $CD8^+$ T cells [22, 35, 39]. For steady-state migration to occur at least some level of DC maturation is required. Following intranasal application of antigen, pulmonary DCs migrating with an apparently mature phenotype mediate tolerance by the induction of IL-10 producing Tr1-like cells [8, 9, 40]. Repeated injections of *in vitro* generated DCs matured in the presence of TNFα prevented the induction of experimental autoimmune encephalomyelitis (EAE) in a murine model, with the induction of antigen specific, IL-10 producing regulatory $CD4^+$ T cells [41]. The term “semi-mature” has been used to describe these apparently mature, tolerogenic, migratory cells [42]. They are mature with respect to upregulation of surface MHC, co-stimulatory and adhesion molecules, but characteristically exhibit low or absent production of pro-inflammatory cytokines.

The maintenance of incomplete signals between T cells and APCs appears to be a key factor in contributing to the induction of tolerance. This phenomena has been indicated even from early reports of co-stimulation blockade [43] and reiterated in more recent work involving altered peptide ligands [44]. Administration of co-stimulatory molecule-deficient or antisense-targeted DCs mediated prolonged car-

diac allograft survival [45, 46], while CD40-deficient DCs suppressed a primed delayed type hypersensitivity response [47]. Inhibitors of the transcription factor NF-κB mediate prolonged allograft survival [48, 49], and NF-κB has been implicated as an important regulator in determining DC tolerance or immunity, at least in part by modulating surface levels of CD40 [47, 50]. It has been reported that $CD4^+CD25^+$ Tregs impact on DC function by down-modulating the co-stimulatory capacity of DCs [51]. A more complete picture of factors contributing to a DC's ability to induce tolerance probably involves not simply a reduction in co-stimulation, but also an alteration in the balance between stimulatory and inhibitory molecules that work in concert to establish activation thresholds and tune cellular responses. Such molecules implicated in DC biology include members of the related leukocyte immunoglobulin receptor (LIR) or immunoglobin-like transcript (ILT) family and paired immunoglobulin-like receptor (PIR) molecules and also activatory and inhibitory FcγRs [52–58]. Production of the tryptophan-catabolizing enzyme, indoleamine-2,3-dioxygenase (IDO), has also been suggested to play a role in DC mediated suppression of T-cell proliferation [59, 60].

The plasticity of DC maturation means that the balance between immunity and tolerance will also be influenced by the overall context of antigen exposure, and it may be that a DC capable of mediating tolerance on maturation in one context might still be able to prime when matured in another. This makes it difficult to generalize about DCs as being universally tolerogenic or immunogenic. However, within any one model or context it should be possible to make comparisons between two sets of DCs with immunogenic and tolerogenic outcomes and thereby draw conclusions on mechanisms that distinguish them.

30.2 Gene Profiling

Comparison of global gene expression patterns provides an essentially unbiased approach to investigating biological phenomena. Unrestricted by current dogma, this type of approach has the potential to reveal previously unanticipated areas for further study and has already proved informative in elucidating aspects of DC biology.

In response to various microbial and inflammatory stimuli a coordinated program of gene expression changes has been revealed that corresponds with the temporal and spatial segregation of different functional aspects of DC maturation [13, 61]. These changes include a transient increase in pro-inflammatory genes, in addition to waves of gene changes required to downregulate phagocytic activity, facilitate migration to draining lymph nodes and to promote T-cell priming, with a previously unappreciated wave of IL-2 expression compatible, with priming of both $CD4^+$ and $CD8^+$ T-cell responses, revealed [61]. Moreover, while it was revealed that a core set of genes change in response to all stimuli, pathogen-specific changes were demonstrated that presumably act to tailor the resulting immune response [13].

DCs matured using the inflammatory mediator TNFα alone, although capable of maturing DCs with respect to upregulation of surface MHC class II and co-stim-

ulatory molecules, cannot drive maturation to a stage necessary for initiation of an optimal immune response. Gene profiling has indicated that TNFα stimulation is insufficient to induce expression of genes encoding pro-inflammatory cytokines, such as IL-6, IL-12p40 and IL-1β, and this is consistent with the designation of these cells as "semi-mature" [42, 62].

Transcript profiling of DCs modulated by a $CD8^{+}CD28^{-}$ T-cell population with suppressive function revealed a downregulation of co-stimulatory molecules, including CD40, CD80, CD86, OX-40L and an upregulation of transcripts regulating NF-κB activity, anti-apoptotic genes and inhibitory molecules, including ILT3 and ILT4 [63]. Transduction of ILT3 and ILT4 into a DC line resulted in cells that, like $ILT3^{high}ILT4^{high}$ APC, induced anergy in $CD4^{+}$ helper T cells and were able to induce the generation of antigen specific $CD4^{+}CD25^{+}$ regulatory T cells, as well as further $CD8^{+}CD28^{-}$ suppressor T cells, in a feedback type manner [56, 63, 64]. Upregulation of ILT3 and ILT4, in addition to other inhibitory molecules bearing immunoreceptor tyrosine based inhibitory motifs (ITIMs), has also been demonstrated on DC populations modulated for tolerance using IL-10, interferon-α or the biologically active metabolite of vitamin D_3, 1α,25-dihydroxyvitaminD3, (VD3) [64, 65], with a particularly potent tolerogenic subset of IL-10-modulated DCs identified as $ILT^{high}BDCA3^{+}$ [65].

The above profiling studies were all facilitated by the availability of *in vitro* culture systems to provide ready access to populations of DCs in ample numbers, at defined stages of activation and at sufficient homogeneity for reproducible analyses. The *in vitro* systems used have either been based on well-defined long-term DC lines, such as D1 and KG-1 [66, 67], or on short-term culture systems developed to generate immature DCs from human monocytes or murine bone marrow [68, 69]. Although profiling of more abundant primary DC populations is possible [70], the time and manipulation involved in isolating these cells is not ideal and the homogeneity of the resulting populations is limited by available sorting and isolation criteria [38]. Using *in vitro* culture systems a number of agents have been reported to modulate the immunostimulatory potential of DCs [50], apparently forcing them to become functionally tolerogenic. These agents include cytokines such as IL-10 [71, 72], TGFβ [73–75] and vascular endothelial growth factor (VEGF) [76], in addition to more traditional pharmacological agents, such as aspirin [77], nicotine [78], LF15-0195 [79], dexamethasone [80, 81] and VD3 [82, 83]. While the exact physiological subset or relative maturation state of DCs responsible for peripheral tolerance *in vivo* remains controversial the generation of tolerogenic DCs using such intervention *in vitro* provides a readily accessible source of potentially regulatory cells for the investigation of tolerance mechanisms using gene expression profiling.

30.2.1 Gene Profiling Technologies

Ideally expression profiling would provide comprehensive information on protein expression patterns, including information on post-translational modifications

and other features that reflect functional activity, but as yet this level of analysis remains beyond the capabilities of proteomic technologies. The alternative is transcript profiling which reveals context-dependent patterns of gene expression, variations in which provide informative predictors of changes in cell function. Several large-scale genomics platforms are currently available for the assessment of gene expression patterns and selection tends to depend both on experimental considerations, such as the requirement for depth of genome penetration and/or the identification of novel genes *versus* analysis of multiple samples, and practical considerations, such as the availability of technology and cost. The most widely used applications are based either on hybridization to known sequences, as in oligonucleotide and cDNA microarray analyses, or on the sampling of transcript information from total mRNA pools. While the approach of expressed sequence tag (EST) enumeration samples partial transcripts, approaches such as serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS™) more efficiently sample short representative transcript tags. Although the depth of transcriptome penetration and procedural automation achieved by MPSS™ make it a very attractive approach (it readily samples millions of short transcript tag sequences in a single machine run) [84, 85], the costs associated with this proprietary technology have so far precluded its more general use. Hybridization-based approaches offer advantages relating to convenience, and the extent of their genome coverage has improved in line with increasing availability of genome data, however this technology is still limited to analysis of known or available transcripts and has proved difficult to standardize across multiple datasets.

30.2.2 Serial Analysis of Gene Expression (SAGE)

SAGE represents a particularly versatile tool for elucidating gene expression patterns. Both quantitative and qualitative transcript information are generated and stored in the form of electronic databases or "libraries" that accumulate as a permanent, accessible resource. Any SAGE library can be readily compared with any other library, or combination of libraries, including those generated in different laboratories, provided the libraries have all been generated using the same anchor enzyme (AE) and are derived from the same species [86]. "Virtual northern" analyses based on SAGE data are now found as a standard component of public transcript/unigene databases. This compatibility across wide data sets is a powerful feature of the technology.

Comparisons of libraries from related but functionally distinct cell populations can be used to focus on transcript changes underlying the functional distinction, while comparison of libraries from cells with related functions can be used to focus on genes responsible for functional similarities. Phenotypically unrelated libraries are often included in comparisons to remove "noise" generated by abundant, widely-expressed "house-keeping" type genes. An expanding resource of libraries (currently including 246 human and 81 mouse *Nla* III libraries) is available at the gene expression omnibus (GEO) data repository at the national center for

biotechnology information (NCBI) and can be downloaded *via* the internet for inclusion in comparative analyses.

Unlike hybridization-based technologies, SAGE samples from the entire mRNA pool with no requirement for prerequisite sequence information and indeed SAGE tags can be used to clone previously uncharacterized transcripts [87]. Depending on the relative positioning, SAGE tags can also distinguish alternatively spliced forms. It has been suggested that 40–60% of multi-exon genes are alternatively spliced, and that of these at least 49% undergo alternative splicing of a terminal exon [88]. While the 3' bias of the original procedure makes it amenable to the cloning and characterization of 3' ends, SAGE has more recently been adapted to generate comprehensive analysis of 5' ends, or alternatively the very extremes of transcripts, a task that has so far thwarted automated genome annotation procedures, providing information on transcription start sites and the alternative use of promoters as well as polyadenylation sites [89–91].

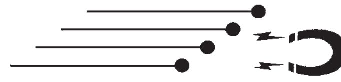
30.2.2.1 **SAGE Methodology**

The original SAGE approach was based on three main principles [92]: Firstly, that a short sequence tag of 9–10 bp is sufficient to uniquely identify a transcript, provided the tag is isolated from a defined position; secondly, that efficient sequencing of these short tags can be achieved by concatenation, provided that there is a means to register the boundaries of each tag; and thirdly, that the number of times a tag is sampled reflects the relative frequency of the associated transcript in the starting mRNA pool. The availability of commercial SAGE kits has now brought this technology within the capabilities of most laboratories, particularly if commercial outsourcing is considered for the large-scale sequencing component.

Typical libraries are generated from 2–5 µg of total RNA, although pre-amplification protocols have enabled libraries to be generated from as little as 40–50 ng [95–98]. RNA integrity is essential for authentic profiling and the quality of each starting RNA population should be routinely established. This can be achieved using as little as 200 pg of material using instrumentation such as the Agilent Technologies 2100 bioanalyzer. A schematic representation of the SAGE procedure is outlined in Fig. 30.2. Modifications from the original protocol [92], reflect streamlining to increase efficiency. Poly A+ mRNA is magnetically isolated using (poly-T)-coated beads with double-stranded cDNA generated directly on the beads. The cDNA is digested using a frequent cutting restriction enzyme (most commonly *Nla* III); this enzyme defines the position within the transcript from which the tag will be isolated and is referred to as the "anchor enzyme" (AE). The most extreme 3′ AE restriction fragments are isolated magnetically, divided in two and unique linkers (1 and 2) ligated onto either half. The samples are then recombined and digested using a type IIS restriction enzyme that recognizes a non-palindromic sequence located towards the 3′ end of each linker and cleaves a defined distance downstream to release a "linker+tag" unit; this enzyme defines the length of the SAGE tag and is referred to as the "tagging enzyme" (TE). The remaining 3′ ends, still bound to the (poly-T)-coated beads, are removed magnetically and the linker-

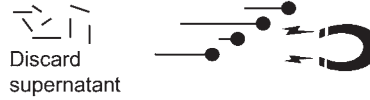
Isolate total RNA | 2 – 5 μg total RNA

Magnetically isolate pA+ RNA & synthesise cDNA



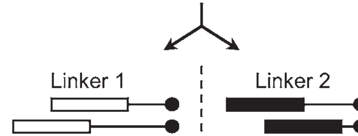
Digest with '*anchor enzyme*' (AE)

Magnetically separate & retain 3' AE restriction fragments



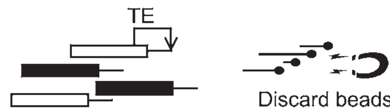
Divide sample

Ligate '*linker 1*' to one half & '*linker 2*' to the other half



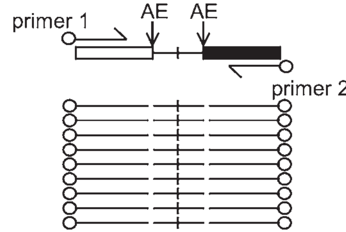
Recombine & digest with a type IIS '*tagging enzyme*' (TE)

Magnetically separate & retain '*linker+tag*' supernatant



Ligate '*linker+tag*' units

PCR amplify (5'-biotinylated primers)



Digest with '*anchor enzyme*' (AE)

ditag

Purify & concatenate '*ditags*'

Size select & clone (pZerO-1, Invitrogen)

96-well PCR screen, PEG precipitate, sequence

SAGE sofware
Extracts ditag sequences

Fig. 30.2 Schematic representation of the SAGE experimental procedure.

tag units purified from the supernatant by precipitation. These units are ligated to generate PCR templates that are amplified using 5′-biotinylated primers located within the linker sequences. "Ditags" are released from the amplified products by again digesting with the AE. Following gel purification of the "ditags", any remain-

ing traces of linker are removed using streptavidin-coated magnetic beads to facilitate efficient concatenation [94]. A commercial PCR clean-up column is used to remove small concatenation fragments prior to size selection and cloning [93]. To facilitate high throughput analysis, cloned inserts are amplified from individual colonies using PCR in a 96-well plate format, the amplified products are precipitated using polyethylene glycol (PEG) and directly sequenced using a single primer. Sequence data is transferred directly to SAGE software (provided by K.W. Kinzler, Johns Hopkins Oncology Center, Baltimore, MD, USA, http://www.sagenet.org), which locates the punctuating AE sites in the concatemers and extracts the intervening "ditag" sequence information. The generation of ditags prior to PCR amplification provides an important safeguard against PCR-generated data distortions and/or inadvertent multiple analysis of the same clone [92]. In theory a 4 bp restriction site should occur on average every 256 bp (4^4), and as such the anchor enzyme (AE) should cleave at least once in every transcript [92]. Although it is appreciated that this assumption is inaccurate it would appear that the number of transcripts missed due to the absence of an AE site is small, <1% [102].

The original tagging enzyme (TE) used for SAGE was *Bsm* F1 which generates tags of ~10 bp (+4 bp contributed from the AE site). More recently TEs that extend further away from the recognition site have been incorporated resulting in "long" and "super" SAGE [99, 100]. *Mme* I cuts 7 bp further away than *Bsm* FI to yield ~(17+4) bp, "long" tags, while *EcoP* 15I generates "super" tags of ~(22+4) bp. So long as the AE is retained in common, these "long" and "super" SAGE libraries remain directly comparable with each other and with the original "short" SAGE libraries over the common AE+10 bp sequence. The "long" tags confer distinct benefits with regard to tag-to-gene annotation, including the ability to map tags directly to the genome, avoiding inconsistencies encountered from the use of incomplete cDNA/Unigene database entries [99, 101]. Justification however for the additional cost and labor involved in sequencing "super" libraries remains to be demonstrated.

30.2.2.2 **Handling Raw SAGE Data**

Data entered into the SAGE software is single-pass and unedited. Low sequence quality will either obscure the serial pattern of AE punctuation or lead to the introduction of ambiguous bases, both of which cause the sequence to be discarded and not entered into the final dataset. Although tags generated as a result of random sequence errors fail to accumulate statistical counts and are ignored, non-random sequence errors arising from highly abundant tags can significantly influence the final data [86, 103]. For "short" SAGE a numerical manipulation excluding tags matching 9 out of 10 bases of any other tag occurring at a >10-fold frequency, has been incorporated to account for accumulated non-random errors [86]. In practice it has been established that once a depth equivalent to 0.1–1% of the most abundant transcript has been achieved (in practice between 10 000 and 30 000 tags) then artifacts accumulate more rapidly than novel gene tags, making additional statistically relevant tag accumulation both inefficient and expensive [86].

The advent of "long" SAGE has essentially resolved issues associated with sequencing errors. The longer tag length has facilitated the incorporation of a cluster algorithm for the development of the corrective software, SAGEScreen, [104]. "Long" SAGE ditag information is extracted from the raw sequence data using SAGE300 software and is passed through the SAGEScreen software to generate an error-corrected tag list.

In an attempt to account for inconsistencies, such as partial entries in cDNA databases, the corrected SAGE tag lists are subjected to an automated hierarchical tag-to-gene annotation process based on Unigene full mapping files and a search algorithm giving priority to a list of hand-annotated entries. For each hand-annotated entry the existence of a plausible polyadenylation sequence has been confirmed, as has the tag location, immediately downstream of the most 3′ AE (Nla III) site. The next annotation priority is given to cDNAs with a polyadenylation signal in the correct orientation, then EST clusters with a probable polyadenylation signal, cDNAs with no polyadenylation signal and finally other matching ESTs. The annotated tag list is imported into a custom-written software package, SAGEClus, that removes specified artifact tags, for example those potentially arising from linkers or primers, and facilitates statistical library comparisons (http://www.molbiol.ox.ac.uk/pathology/tig/software/softlist.html) [86].

30.2.3 Accumulation of a Comparative SAGE Resource for Identifying Tolerance-associated Genes

The capacity of SAGE to make comparisons across wide data sets offers particular potential to correlate gene expression with functional phenotype. By comparing immune and non-immune cell populations, individual genes and "signatures" of genes associated with pro-tolerogenic rather then immunogenic phenotypes can be elucidated to provide much needed novel markers of immune regulation and mechanistic insight into the body's natural tolerance machinery.

For informative library comparisons the authenticity and homogeneity of the starting cell populations must be well established. A culture system adapted from that previously described by Inaba et al. [69] has been used to generate a series of functionally distinct murine bone marrow derived DC populations. Following 7 days culture in GM-CSF cells exhibit surface and functional phenotypes characteristic of immature DCs, while the inclusion of lipopolysaccharide (LPS) for the final 18–20 h causes them to acquire characteristics typical of mature DCs; that is increased surface expression of MHC class II and co-stimulatory molecules (CD40, CD80 and CD86), diminished capacity to take up and present new antigen and enhanced capacity to stimulate naive T cells. DC populations grown under these conditions are >90% homogeneous with respect to expression of CD11c and SAGE libraries have been generated from both the immature and mature states [87].

Consistent with previous findings, bmDCs counter-modulated by each of the three pharmacological agents IL-10, TGFβ and VD3, (as outlined in Fig. 30.3), are reminiscent of immature bmDC, with moderate to low MHC class II and low lev-

Fig. 30.3 Generation of bmDC populations for SAGE analysis. Cells were prepared using an adaptation of the culture system previously described by Inaba *et al* [69, 87]. Immature cells were harvested on day 7. IL-10 was added (20 ng ml^{-1}) from day 6 and the cells harvested on day 9. Alternatively, 1α,25-dihydroxyvitamin D3, VD3 (10^{-7} M) was added from day 3, or TGFβ (2 ng ml^{-1}) added from day 0, with the cells harvested on day 7. When added, LPS (1 μg/ml) was included for the final 18–20 h of culture.

els of surface co-stimulatory molecules. However, unlike immature bmDCs the levels of these markers do not increase in response to LPS and the modulated cells are impaired in their ability to generate IL-12p70 and to support naïve T-cell proliferation [72, 87, 105–109]. The modulated cells were also tested for their ability to mediate tolerance in a male antigen specific, murine TCR transgenic skin transplant model [110]. While the administration of LPS-matured male bmDC prior to grafting did not affect rejection of a male graft, administration of modulated DCs, cultured with or without the addition of LPS, mediated graft survival (unpublished data). SAGE libraries have been generated from bmDCs modulated with IL-10, TGFβ and VD3, and from IL-10-modulated cells treated with LPS.

An accumulated resource comprising almost fifty murine SAGE libraries has now been generated [86, 87, 111]. In addition to libraries generated from the untreated and pharmacologically-modulated bmDC populations, with or without the addition of LPS, described above, the resource also includes libraries derived from embryonic stem (ES) cells and DCs differentiated from these cells, again with and without maturation in response to LPS [112, 113], purified T-cell populations and polarized T-cell clones, including defined "adaptive" Tr1-like and "natural" $CD4^{+}CD25^{+}$ regulatory populations [86, 114–116], in addition to a B cell population and a number of non-immune related populations, such as a fibroblast line and organs such as the heart and brain. Both "long" and "short" SAGE libraries are represented, and new libraries are continually added to the resource as they become available. For the purpose of SAGE, house-keeping genes are defined as genes corresponding to tags with significant expression in all the libraries of the resource and for clarity are removed from many of the analyses.

An overview of processes involved in using the SAGE resource to identify and follow up on tolerance-associated genes is shown in Fig. 30.4. The hand-annotated gene list used for the hierarchical tag-to-gene annotation process was initially established to ensure accurate assessment of immunological genes of interest, however as unknown tags are assigned or automated assignments confirmed they are also added to this list. Statistical, pairwise and global library comparisons are facilitated by SAGEClus software [86]. Pairwise scatter plots, associated with automated annotation links, readily identify genes differentially expressed between two

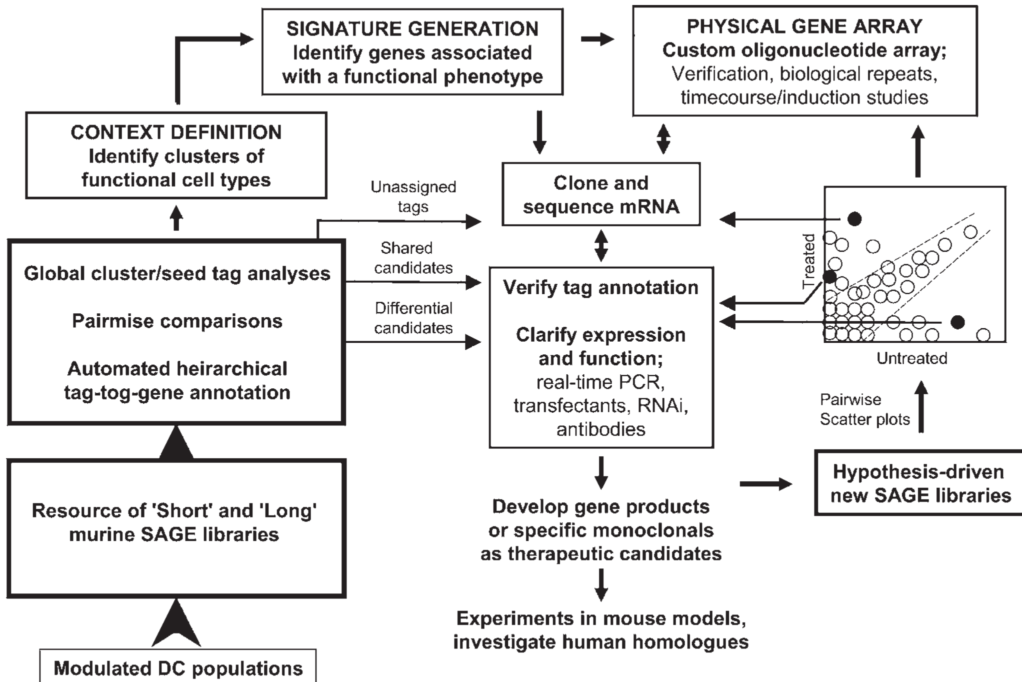


Fig. 30.4 A generalized strategy for the identification and analysis of candidate genes associated with tolerance. This strategy relies on comparative analysis of an accumulated resource of murine SAGE libraries (anchor enzyme *Nla* III).

populations, such as tolerogenic and non-tolerogenic. Global cluster analyses provide a measure of the similarity/relatedness of different cell populations with respect to their gene expression patterns, and can identify clusters of genes associated with related populations. Alternatively "seed" tags exhibiting idealized hypothetical expression profiles that mirror a particular phenotype, e.g. tolerogenic or non-tolerogenic, can be introduced and tags with the closest matching profiles identified. Combinations of these methods can be used to highlight groups of tolerance associated candidate genes, or "signatures". Signature genes can be brought together on a custom oligonucleotide microarray to facilitate rapid simultaneous analysis in various different samples, including biological replicates of the populations used for the SAGE, kinetic time-course or induction studies and to investigate the representation of the candidate genes in samples from in vivo investigations.

The annotation of each candidate tag is verified by hand. When no unique transcript can be identified or the short tag assignment is inconclusive then the "long" SAGE tag can be used to query the mouse genome directly. If the assignment is ambiguous, then the corresponding transcript can be revealed using rapid amplification of cDNA ends (RACE) procedures [117]. Differential expression patterns are clarified by real-time quantitative PCR methods.

Where the candidate gene is known, reagents such as antibodies may be available and biological information accessible from the literature. For less well characterized/unknown sequences computational analyses can reveal potential open reading frames and gene/protein homologies, as well as information regarding structural and functional features of the putative protein that may provide insight into cellular location and function. Biological tools such as antibodies and chimeric constructs, the expression of mutant or dominant negative forms, or the use of gene silencing and RNAi knock-down technologies, can be further utilized to unravel biological relevance. Custom SAGE libraries can be generated to reveal molecular mechanism by which specific genes affect regulation. As therapeutic targets are revealed human homologues can be identified and investigated.

30.2.3.1 Relationship of Modulated DC Populations based on Gene Expression Patterns

The relationship of different cell populations based on their relative gene expression patterns can be established using clustering programs within the SAGEClus software package [86]. Using this analysis, DC libraries represented within the current SAGE resource cluster together, away from a small cluster of T cell-related libraries and a further small cluster of embryonic cell libraries (Fig. 30.5). As expected the two independently generated “long” and “short” LPS matured bmDC libraries cluster together.

Within the DC cluster, the non-immunogenic libraries, generated from populations that were unable to stimulate proliferation of naïve T cells and which mediated tolerance when transferred to the TCR transgenic skin transplant model, clus-

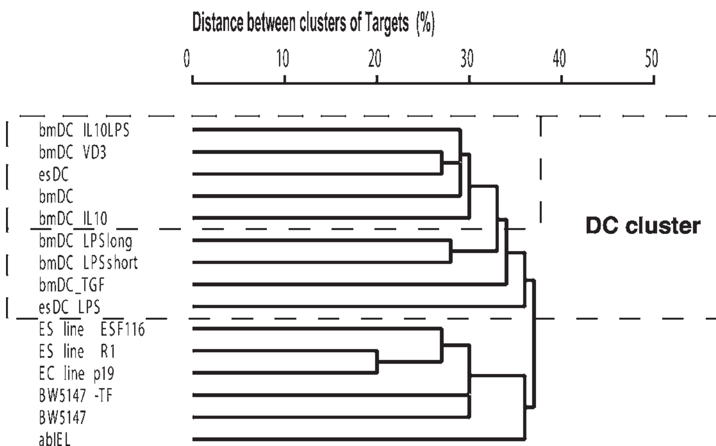


Fig. 30.5 A dendrogram illustrating the relative relationship of immature, LPS-matured and modulated DC populations based on their relative gene expression patterns. Clustering was performed using SAGEClus software [86]. (Libraries downloaded from public sites; R1-ES cell line (GSM580), EC line p19 (GSM1682), $\alpha\beta$ intra-epithelial lymphocytes (IEL) [118].)

ter together; that is immature bmDC and ES-derived DC, IL-10- and VD3-modulated bmDC and bmDC modulated with IL-10 and subsequently exposed to LPS. The exception is the TGFβ-modulated library which clusters independently (Fig. 30.5). This would imply that the mechanisms by which TGFβ invokes immune-modulation are at least to some degree distinct from that of IL-10 and VD3.

30.2.3.2 **Elucidation of "Signatures" of Genes Associated with Tolerance**

Introducing hypothetical "seed" profiles into the SAGEClus analysis can be used to identify clusters of genes whose patterns of expression correlate with a functional phenotype, such as tolerance inducing capability. Libraries are selected as "containing", "not containing", or "no preference", and tags matching the selected profile are listed in order of compliance with the selected profile (Fig. 30.6).

Tags associated with pharmacologically-modulated DC populations, but not with immunogenic LPS-matured bmDC or esDC populations have been identified using a hypothetical test pattern and the most closely matching tags are shown in Fig. 30.6. The alternative use of polyadenylation sites generates two tags for the chemokine CCL6, both of which are represented in this cluster. Consistent with the SAGE data, it has previously been reported that CCL6 expression is not induced in response to LPS, but is increased in response to the cytokines IL-3, IL-4 and GMCSF [119]. CCL6 is particularly chemotactic for monocytes and macrophages and significantly enhances macrophage phagocytic activity [120, 121]. It belongs to a subgroup of chemokines that each contain a unique second exon and there is evidence that, like other members of this subgroup, CCL6 mediates at least some of its functional activity through CCR1 (the tag for which is also represented in this modulated DC gene cluster) [122]. While CCR1 is known to mediate recruitment and maintenance of immature DC at inflamed sites, exposure to IL-10 causes this receptor to become functionally uncoupled and to take on a ligand scavenging role [123]. It is unclear what the functional state of this receptor is in other modulated populations although the relative expression of CCL6 in the selected DC populations has been confirmed by real-time quantitative PCR and western blot analyses (unpublished data).

TGFβ-induced 68-KDa protein, also known as beta Ig-H3, is an extracellular matrix (ECM) protein that mediates cell adhesion and migration through interactions with integrins and may play a role in regulating angiogenesis [124]. Embigin is also implicated in cell binding *via* integrins [125], while ring finger protein-130 is a zinc-finger protein, cloned from a myeloid precursor line following removal of IL-3 to induce apoptotic death [126]. Transglutaminase-2, Tgm2, belongs to a family of molecules that catalyze post-translational modifications that result in polymerized, cross-linked proteins. It functions both intracellularly and extracellularly and has been implicated in stabilization of the ECM [127]. Tgm2 would appear to play an important role in regulating the bioavailability of TGFβ, as only after Tgm2-catalysed linkage to the matrix does latent transforming growth factor binding protein-1 release active TGFβ [128]. It has also been implicated in cytoskeletal polymerization during the final steps of apoptosis, preventing the release of cell com-

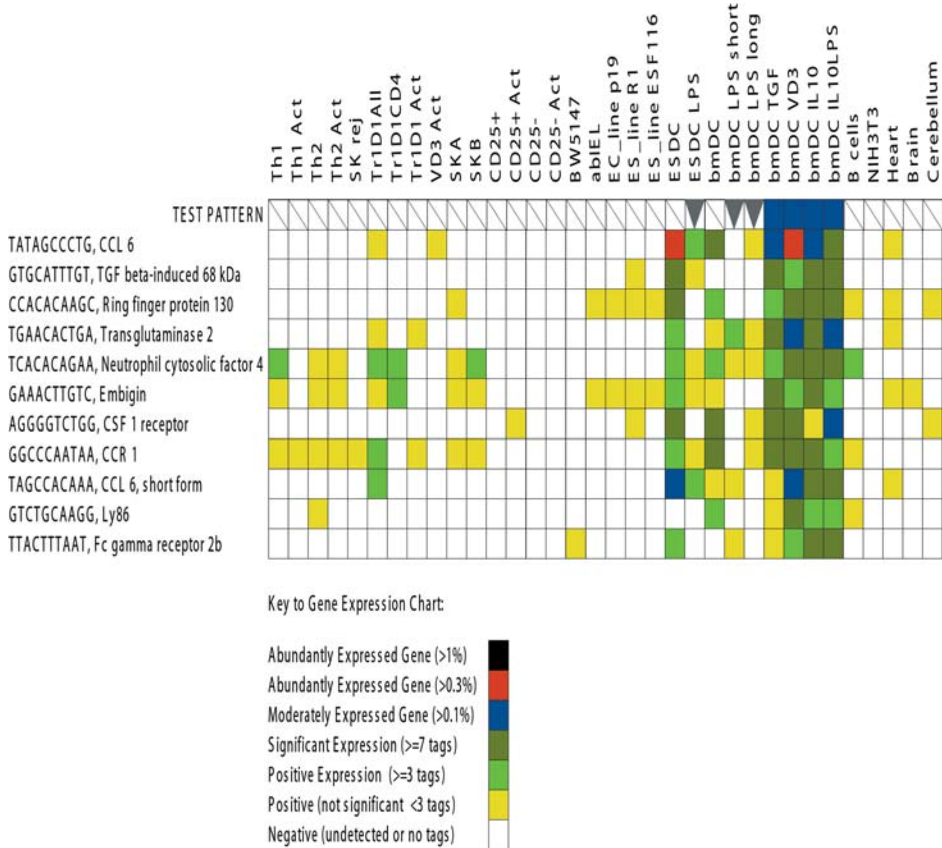


Fig. 30.6 SAGE tags associated with modulated DCs. Using SAGEClus software, tags associated with the pharmacologically-modulated DC populations have been selected based on the relatedness of their expression profiles to the idealized test pattern indicated in the top row of the clustergram. A blue square in the test pattern indicates moderate tag representation in that library, as indicated in the expression key, while an inverted grey triangle indicates no tag representation and a diagonal line no preference. Details of the comparator libraries indicated can be found in [86].

ponents that may give rise to inflammatory or autoimmune responses, and in the process of receptor-mediated endocytosis [129]. Neutrophil cytosolic factor-4 is otherwise known as p40phox and is a regulatory component of the NADPH oxidase complex that is activated during phagocytosis to mediate the generation of mirobicidal antioxidants [130]. Signaling through the colony stimulating factor-1 receptor, CSF1 receptor, regulates the survival, proliferation and chemotaxis of macrophages and supports their activation [131]. MD-1 (Ly86) has been implicated in mediating responses to LPS. As MD-2 is required for the surface expression of TLR4, so the related protein MD-1 associates with the protein RP105, and mice lacking either MD-1 or RP105 have been shown to exhibit a decreased immune response

against LPS [132, 133]. Despite this, structural features of MD-2 required for binding of LPS are absent from MD-1 and it has recently been proposed that signals transduced through RP105/MD-1 by an unknown ligand may somehow act collaboratively on the LPS-activation pathway through TLR4/MD-2 [134]. Finally shown in this limited cluster is the tag corresponding to the inhibitory Fcγ receptor. Selective blockade of this receptor leads to spontaneous and full maturation of DCs, requiring the presence of endogenous plasma IgG and it is suggested that the activation status of DCs in normal human serum depends on the balance between activating and inhibitory FcγRs [58].

While the functional relevance of these genes requires experimental clarification, such signatures can also serve as a useful diagnostic to facilitate the identification of functionally related populations or to monitor the appearance of tolerogenic DCs *in vivo* using custom microarray analysis as described below (Section 30.3.1).

30.2.3.3 **Identification of Novel Genes**

By sampling tags from the entire transcriptome SAGE provides for the possibility of identifying and cloning previously undescribed transcripts. The pro-inflammatory chemokine DCIP-1 was first revealed as a 10 bp SAGE tag, represented in LPS-matured, but not immature bmDC libraries, for which no annotation could be made from the existing databases [87]. The corresponding transcript was subsequently cloned, initially using the tag itself to prime amplification of the 3′ end. The novel transcript revealed an ELR^+ CXC chemokine, that by homology is a novel member of a subgroup of pro-inflammatory chemokines, comprising murine CXCL1 (MIP-2) and CXCL2 (KC), the rat CINC and the human GRO proteins. It was subsequently demonstrated that this chemokine is a ligand for CXCR2, and that it mediates selective recruitment of neutrophils *in vivo*. The kinetics of induction of the DCIP-1 transcript are consistent with expression during the innate pro-inflammatory phase of DC maturation.

30.2.3.4 **SAGE Library Comparisons Provide Insights to Biological Mechanism**

Although bmDC modulated with IL-10 do not acquire the ability to stimulate naïve T cells in response to LPS, and similarly do not increase their levels of surface MHC class II or co-stimulatory molecules, the global changes in gene expression patterns observed by multiple pairwise scatter-plot comparisons are immediately supportive of a mechanism more subtle than simple blocking of maturation. On addition of IL-10 to bmDCs, ~23% of the tag changes were also observed in response to LPS, suggesting that at least some features of IL-10 modulation are actually in common with the LPS maturation process. After LPS treatment of the IL-10-modulated DCs, ~37% of the tag changes were in common with those mediated by addition of LPS to unmodulated bmDC, and this represented ~56% of the unmodulated bmDC response to LPS [87]. While classic maturation of IL-10-modulated bmDCs does not occur, aspects of the normal response do appear to be retained.

Closer inspection of individual tag modulations revealed that although CCR7 SAGE tag levels were markedly increased in response to LPS, this increase was not seen in bmDCs previously modulated by exposure to IL-10. A similar block was also observed in the appearance of SAGE tags for the chemokines CCL17, CCL21 and CCL22 that promote various DC:T cell interactions, suggesting that the ability of DCs to recruit adaptive immunity in response to a microbial stimulus is hindered as a consequence of IL-10 modulation [87]. Indeed it has been demonstrated that IL-10 does inhibit maturation-induced migration of DCs to lymph nodes by blocking the switch from inflammatory chemokine receptors, such as CCR1 and CCR5, to the lymphoid homing receptor CCR7, [123, 135]. In mice infected with *Leshmania donovani*, IL-10 mediated inhibition of CCR7 expression results in a spatial segregation of DCs and T cells that contributes to immune-suppression and promotes pathogenesis [136]. Tag levels for the chemokine receptors CCR1 and CCR5 were not reduced in the IL-10-modulated DCs in response to LPS, consistent with reports that these receptors are not downregulated following IL-10 modulation, but functionally uncoupled, acquiring a scavenger role and possibly contributing to subsequent dampening of potentially destructive inflammation [123].

Multiple pairwise SAGE comparisons combined with quantitative real-time PCR data, indicated that in contrast to their impaired ability to recruit adaptive immunity following exposure to microbes, IL-10 modulated DCs are not only not impaired, but indeed appear to be enhanced in their ability to mount innate inflammatory responses [87]. Transcription of the genetically-linked inflammatory chemokines DCIP-1, CXCL2, CXCL4 and CXCL5, involved in neutrophil recruitment and activation, and the pro-inflammatory cytokine IL-1, are all increased in IL-10-modulated DC in response to LPS, while a decrease in tag numbers for the decoy-receptor IL-1R2 in response to IL-10 alone is suggestive of priming for IL-1 responsiveness. An increase in tags for both membrane-bound and soluble innate pattern recognition molecules, such as MARCO, CD14, TLR2 and galectin-3, in combination with a general increase in tags corresponding to degrading lysosomal enzymes, is suggestive of an enhanced potential for phagocytic bacterial clearance in LPS-treated, IL-10-modulated DCs, consistent with observations of increased antigen uptake by IL-10-treated DCs in the presence of bacteria [87, 137]. These observations are in contrast to reports of IL-10 as an anti-inflammatory cytokine and probably reflect the importance of context in determining the outcome of cytokine exposure.

The increase in inflammatory potential resulting from exposure to IL-10 appears to be tempered by a co-ordinate increase in the production of anti-inflammatory agents [87]. Tags corresponding to hemoxygenase-1, reportedly responsible for IL-10-mediated protection of mice from LPS-induced septic shock [138], and arginase-1, which competes with iNOS for the substrate arginine, downregulating the production of NO [139], are both increased in the IL-10-modulated, LPS-exposed library. Carbon monoxide generated by hemoxygenase-1 breakdown of heme also inhibits T-cell proliferation [140, 141]. Tags for the chemokine CXCL7 were uniquely acquired to abundant levels in the IL-10-modulated DC library and this was reversed following the addition of LPS [87]. Differential rates of processing of

various precursor forms of this chemokine result in pro- and anti-inflammatory scenarios [142]. Inflammatory responses reflect a complex network of chemokine/protease interactions and while the substantial increase in cathepsins D, S, L, B and C may be related to increased antigen processing in the IL-10-modulated DCs, such proteases could also mediate as yet unrealized aspects of the IL-10-modulated DC phenotype, including roles in inflammation, cell migration and transcriptional control of the cell cycle [143–145].

The outcome of antigen presentation to T cells is at least to some extent determined by a balance of inhibitory and stimulatory receptor ligand interactions. An important contribution to the IL-10-mediated decrease in DC stimulatory capacity is provided by the interaction of PD-L1 and PD-L2 with their cognate inhibitory receptor PD-1, expressed on T cells [146]. This was reflected in the SAGE data by increased levels of the PD-L1 tag in the IL-10-modulated SAGE library and in particular in the IL-10-modulated library exposed to LPS [87]. PD-1 expression is reported to be more strongly induced on T cells that have received a weak antigenic signal implying that interactions with PD-1 will preferentially inhibit low avidity antigen receptors [146, 147].

30.3 Downstream Assessment of Tolerance Associated Candidate Genes

The immediate action on identifying a candidate tag is to verify the automated gene assignment and to clarify the authenticity of the transcript candidacy. While verification of the assignment can frequently be achieved by critical reference to existing databases, it can also on occasions require experimental clarification using 3′ RACE, to ensure the assignment is indeed derived from a full-length transcript. The transcript expression pattern is verified using quantitative real-time PCR analysis, which can also provide information on the relative distribution of alternatively spliced versions of a candidate indistinguishable by SAGE.

30.3.1 Simultaneous Assessment of Multiple Candidate Gene Expression Levels using a Custom “Immunochip”

Limited commercial microarrays have been used to screen biological replicates to validate a number of the SAGE libraries included in the SAGE resource. A further application of microarray technology is being adopted through the generation of an “in-house” custom “immunochip”. SAGE has implicated multiple candidate tags, corresponding to genes of known and unknown function, as having a potential role in mediating tolerance. Oligonucleotides representing these candidates, as well as candidates identified from parallel SAGE studies investigating regulatory T cells and also candidates reported in the literature, are being combined onto a custom array. It is envisaged that this “immunochip” will be used to facilitate rapid, simultaneous analysis of genes of interest across a range of different samples and will thus allow assessment of the kinetics of induction or repression of candidates

by time-course studies and the relative expression of candidates in samples obtained from *in vivo* tolerance models. The convenience of arrays, combined with the inclusion of SAGE-derived diagnostic gene “signatures”, should facilitate a more speculative use of the “immunochip”, such as during the preliminary stages of an experimental investigations to consider the effects of variations of culture conditions or treatment regimes on the induction of tolerance.

30.3.2
Assessing the Functional Relevance of Tolerance Candidates by Genetic Manipulation of DCs

Expression profiling and gene discovery form only the first steps in elucidating molecular mechanisms of tolerance. The ultimate challenge is to establish informative functional assays in order to investigate downstream biological relevance. This process has proven particularly difficult in the DC field, largely due to the inherent resistance of primary DCs to the introduction of heterologous genes, their susceptibility to mature upon manipulation and their short life span following terminal maturation. While efficient transfer of DNA constructs to primary DCs has been reported using a number of viral vectors, the transduced cells are often perturbed in their function and/or limit investigations to certain stages of DC development [148, 149].

Although tools such as antibodies and chimeric constructs can be used to investigate the contribution of candidates to tolerance, genetic manipulations, such as over-expression or expression of mutant/dominant negative forms and the use of RNAi knock-down approaches, are essentially precluded in primary DCs. An alternative has been presented by the demonstration that DCs can be differentiated in culture from ES cells [113]. These embryonic stem cell-derived DC (esDC) undergo significant expansion *in vitro*, are phenotypically stable over time and retain the capacity to mature in response to LPS [111–113]. Genetic manipulations can be achieved at the level of the ES cell using standard methodologies, avoiding the inherent difficulties associated with manipulating primary DCs, or the investment of generating manipulated mice as a source of bone-marrow from which to generate genetically modified DCs. The feasibility of generating stable lines of genetically-modified esDC has been verified using EGFP [112]. The stable EGFP-esDC lines maintained their capacity for maturation in response to LPS with no associated loss of transgene expression and retained their migration patterns *in vivo*. The capacity of different candidate transgenes to modulate or skew this process of maturation *in vitro*, in combination with investigation of the effects of these modified cells in an *in vivo* transplant tolerance model (see below) will be informative as to their functional role in the balance of immunity *versus* tolerance.

Comparative SAGE analysis of the parent ES cell line and differentiated esDC, has provided further supportive data for the integrity of this esDC system [111]. These SAGE libraries are available for subtractive comparisons and can be used to identify genes responsive to a transfected candidate transcript. The feasibility of long-term stable expression of knock-down hairpin RNAs in this system is currently under investigation.

30.3.3
Assessing the Functional Impact of Candidates in an in vivo Tolerance Model

A murine TCR transgenic skin transplant model has been established to assess the tolerogenic potential of manipulated DC populations and of genetically-modified esDC. Female CBA/Ca.A1.RAG1$^{-/-}$ mice possess T cells that are specific for a male peptide, HY, presented in the context of H-2E^k, and provide a model of transplant rejection mediated by CD4$^+$ T cells [110]. The RAG1$^{-/-}$ background ensures the monospecificity of this model and allows for a reductionist approach to be taken to the investigation of conditions influencing the establishment of T-cell tolerance. In addition to lacking CD8$^+$ T cells and B cells, these RAG1$^{-/-}$ mice also notably lack naturally occurring CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. While control female CBA/Ca.A1.RAG1$^{-/-}$ mice receiving PBS alone all promptly reject male skin grafts with a mean survival time of 14 days, tolerance has been demonstrated in this system through prior treatment with a blocking antibody directed to CD4 [5], using an altered-peptide ligand [44] and more recently, by the injection of immature and pharmacologically-modulated male DC populations (unpublished data). The tolerance induced is antigen-specific, indefinite and dominant and involves an accumulation of FoxP3$^+$ regulatory T cells in the accepted graft. By administering genetically-modified male CBA/Ca-derived esDCs prior to skin grafting the impact of individual candidate molecules on graft survival and the induction of tolerance can be assessed.

30.4
Downstream Clinical Relevance

An incentive driving investigations into tolerance mechanisms is the potential to facilitate improved clinical outcome in situations of dysregulated immunity, such as allergy and autoimmunity, and in the more contrived situation of transplant rejection, where current drug therapies are both relatively non-specific and require long-term, repeated administration, whilst conferring a multitude of deleterious side effects. The ultimate goal is to develop short-term therapies that harness natural tolerance mechanisms to mediate long-term, antigen-specific re-education of the immune system. For application in the clinic this re-education will have to be achievable in mature immune systems and in the case of allergy and autoimmunity, in systems already primed to respond to the deleterious antigen.

In pharmacological terms, the use of drugs that block pathways in DCs critical to the activation of effector T cells, whilst sparing elements that vaccinate Tregs is a clear target direction for the future and could yet prove decisive in the much-heralded era of stem cell-derived organ replacement therapies. Although acute rejection can be avoided by the generation of organs devoid of DCs, a more robust solution, also encompassing issues of chronic rejection, may be to pre-tolerize by inoculation with immature esDCs, or esDCs manipulated for tolerance, that are derived from the same parent ES cell line as that used to derive the replacement organ itself [150].

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Part B
Dendritic Cells in Disease

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XI
Parasites

31
Malaria

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31.1
Introduction to Malaria

Malaria remains the world's worst public health problem after HIV/AIDS. Globally 2.4 billion people (40% of the world's population in 1999) from more than 90 countries of the world are at risk of being infected and up to 500 million suffer from the disease with varying degree of severity. It is estimated that malaria kills 1 million people annually, mainly children below the age of 5 years and pregnant women [1, 2]. Almost all of these deaths are caused by *Plasmodium falciparum*, one of the four species of malaria parasites that infect humans. Other species infecting humans are *P. vivax*, *P. malariae* and *P. ovale*. This high burden of mortality is not evenly distributed but falls heavily on sub-Saharan Africa, where over 90% of malarial deaths occur and it is estimated that over 5% of children die before their fifth birthday [3] . Malaria is also a primary cause of poverty as a consequence of factors that include effects of the disease on fertility, population growth, saving and investment, worker productivity, absenteeism, premature death and medical costs [4]. Malaria-specific mortality has more than doubled in the last two decades and the situation continues to worsen due to wide spread resistance of plasmodium parasites to preventative and therapeutic drugs. It is estimated that malaria lowers the economic growth of affected countries by 1.3% of their gross domestic product [5] thus contributing to the poverty of these already impoverished communities.

P. falciparum has a complex life cycle with many different developmental stages in the mosquito vector and human host. The bite of an infected *Anopheline* mosquito injects infective sporozoites into the human host, where they are transported rapidly via the blood stream to hepatocytes in the liver. Within the hepatocyte, the parasite matures, differentiates, and undergoes one round of asexual multiplication forming approximately 20 000–40 000 haploid merozoites that are released

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into the blood-stream. This stage takes about 7 days and does not give rise to clinical symptoms. The released merozoites immediately invade red blood cells (RBC) and undergo a process of growth and asexual multiplication to produce between 8 and 32 daughter merozoites per every infected erythrocyte over a period of 48 h. When the daughter merozoites are fully matured (the schizont stage), the infected red cell bursts, releasing the merozoites to invade other erythrocytes. This period of exponential growth is responsible for all the clinical symptoms of malaria and continues until the parasite multiplication is controlled by drug treatment, the immune response, or death in some cases. A small proportion of the invading merozoites undergo an alternative pathway of differentiation and develop into either male or female gametocyte, which are subsequently taken up in a mosquito blood meal. In the mosquito mid-gut, the male and female gametes fuse to form a zygote, which then undergoes a series of complicated differentiation, and growth stages that results in the production of infective sporozoites in the salivary glands of the mosquito.

31.2
Antigenic Variation

Infected RBC of *P. falciparum* (Pf-iRBC), containing the late trophozoite and schizont stages (24–48 h post-invasion) sequester on endothelial cells in almost all tissues. Deep tissue sequestration of Pf-iRBC is generally believed to favor the survival of parasites by preventing Pf-iRBC passage through the spleen where they would otherwise be recognized as abnormal and removed. Sequestration in deep tissue microvasculature may also promote rapid asexual multiplication by placing the Pf-iRBC in a parasite-favoring micro-aerophilic environment.

Sequestration is mediated by adhesion of Pf-iRBC to a variety of host receptors expressed on endothelial cells, RBC and leukocytes (reviewed in [6]). Almost all field and laboratory isolates bind to CD36 and some of them can bind in addition to CD31, CD35 and CD54. Pf. iRBC insert parasite-derived proteins into the RBC membrane, the *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1). PfEMP-1 is encoded by a multigene family of approximately 60 *var* genes per haploid genome [7]. *Var* genes are highly diverse but share a similar organization. They contain two to five copies of a motif denoted “duffy binding-like ligand” (DBL). A second motif common to all PfEMP-1 is the cysteine-rich interdomain region (CIDR) of which there are one or two copies termed α and β. The first CIDR, CIDR-1α is found immediately after DBL1α in most *var* genes and together they form a semi-conserved head structure [8]. Adhesion to different host receptors has been located to distinct domains expressed by different var genes. For instance, CIDR1α binds to CD36 although not all CIDR1α harbor this property [9]. Apart from PfEMP-1, three additional highly variable families of *P. falciparum* proteins have been described: riffins, STEVOR and clag-9 [7]. Their functional properties are not well described yet.

31.3
Animal Models for Malaria

Several Plasmodium strains infecting the free living rodent species *Grammomys surdaster* and *Thamnomys rutilans* have been adapted to infect laboratory mice. None of these models can replicate all the aspects of disease as observed in human malaria but they are used to investigate specific aspects of the immune response to malaria parasites. Although iRBC of rodent Plasmodium species can sequester to a certain extent, particularly in the liver and the spleen, sequestration is not as pronounced as in Pf-iRBC and the parasite molecules that mediate adhesion have not been identified. However, like *P. falciparum,* rodent Plasmodium species encode variant surface proteins, which may be important for the evasion of humoral immune responses [10]. Immunity to blood-stage infection seems to vary with particular host–parasite combinations. For example, *P. yoelii* and *P. berghei* infections are not controlled in B-cell-depleted mice [11, 12] while *P.chabaudi chabaudi*, *P.chabaudi adami* and *P. vinckei* are partially controlled [13–16] suggesting that, although antibodies are critical for clearing infections, different parasite species vary in their ability to mount cellular-mediated mechanisms. While animal models provide valuable insights into immune response mechanisms during the course of one infection, they lack the extensive chronicity and exposure-related acquisition of immunity typical for human infections with *P. falciparum* . Therefore, studies in animal models have to be complemented by studies in human *P. falciparum* infections involving both *in vitro* and *ex vivo* systems coupled with immuno-epidemiological studies in endemic areas.

31.4
Acquired Immunity to Malaria

Despite decades of research, naturally acquired immunity to malaria is still poorly understood. *P. falciparum* infection rarely if ever induces sterile immunity but repeated infection eventually results into clinical immunity with moderate to no clinical symptoms and reduced mortality. In areas of intense, perennial transmission, the main load of malarial disease is experienced by children. Here, a certain degree of clinical immunity to severe, life-threatening malaria is apparent after a few disease episodes and is essentially complete by the age of five years [17]. However, the vast majority of adults living in endemic areas may have low levels of circulating parasites and yet few will have mild, if any, clinical symptoms. Many targets of both T-cell and B-cell responses are either polymorphic or variant antigens. Therefore, infection with different strains or expression of a different variant during chronic infection requires that a new immune response to the polymorphic or variant antigen is mounted. This will occur on the background of already existing immune responses and therefore take a different course from a primary infection. Protective immune responses in clinical immune individuals are the summary of

responses to different polymorphic or variant antigens rather than a response to one particular antigen.

31.4.1
Immune Response to Liver Stages

It is not known whether sterile immunity to the liver stage of infection occurs under natural conditions although irradiated sporozoites can protect both mouse and man from challenge with homologous parasite strain and induce some cross protection against heterologous parasite strains. Evidence from mouse models suggests that liver-stage responses are primarily mediated by CD8 T cells. *In vivo* depletion of CD8 T cells completely abrogates protection, while adoptive transfer of CD8 T cells to naïve mice confers protection [18, 19]. Furthermore, β-2-microglobulin$^{-/-}$ mice, which lack MHC class I are not protected by either active immunization or passive transfer of wild type, splenic T cells from immune mice [20]. It is now well established that protection in mice induced by immunization with irradiated *P. berghei* [21, 22] or *P. yoelii* [23] sporozoites is absolutely dependent on CD8 T cells, IFN-γ and nitric oxide (NO). More recently, IL-12 and NK cells involvement in this mechanism has been demonstrated [24].

The liver stage of the *P. falciparum* infection is now an important target in experimental vaccines [19]. The efficacy of subunit vaccines is now being tested in challenge studies under experimental settings in the UK and the USA, as well as under natural transmission in endemic areas [25]. Some vaccines tested in nonimmune volunteers or in endemic areas showed reduction in infected hepatocytes, infection rates or disease severity at least for a short period of time [26, 27].

31.4.2
Cellular Immunity to the Erythrocytic Stage

During the erythrocytic stage, the malaria parasite spends most of its time in RBCs. RBC do not express appreciable levels of either MHC class I or class II and it is therefore unlikely that parasite antigens would be presented by iRBC to induce T-cell activation. However, antigen presentation by dendritic cells (DC) and macrophages will result in T-cell activation which will either have a direct or indirect effect on the control of parasitemia. Most of the available evidence for a role of T cells in blood-stage malaria comes from animal models and *in vitro* T-cell stimulations using peripheral blood mononuclear cells (PBMC) from people living in malaria endemic areas.

It is clear from murine malaria models that CD4 T cells provide help to B cells to make protective antibodies. B-cell-deficient mice failed to control *P. yoelii* parasitemia [11], and protection from challenge in naïve-irradiated mice could be transferred by a mixture of immune CD4 T and B cells [28]. In the *P. c. chabaudi* and *P. c. adami* models, infection can be controlled in mice depleted of B cells [14, 16, 29, 30]. During the acute phase, the responding T cells are predominantly Th1 which are thought to act by inducing cell-mediated parasiticidal mechanisms followed by

a shift to a Th2 phenotype reflecting the importance of CD4 T-cell help to B cells in order to make antibodies to eliminate parasitemia [31, 32].

There is evidence that T cells from malaria-exposed donors proliferate or produce cytokines in response to malaria antigens (reviewed in [33]). A limited number of studies have reported *in vitro* data using T/B-cell cooperation assays suggesting that CD4 T cells give help to B cells resulting in antibody production [34, 35]. There is also limited evidence that CD4 T cells may have an effector role beyond giving help to B cells in malaria by inhibiting parasite growth *in vitro* [36].

Further evidence for the involvement of CD4 T cells in naturally-acquired immunity to malaria comes from epidemiological studies on the interaction between malaria and HIV-1. HIV-1 has been associated with increased frequency of clinical malaria and parasitemia in an adult cohort in Uganda [37, 38], and pregnant women in Malawi [39–41] and Kenya [42, 43]. In each of these studies, this association was more pronounced with increasing depletion of CD4 T cells. Collectively, these data underline a critical role for CD4 T cells in mechanisms that mediate antimalarial immunity during the asexual blood-stage cycle in people who are naturally exposed to endemic malaria.

31.4.3
Humoral Immunity to the Erythrocytic Stage

Perhaps the strongest evidence yet that antibody has an important anti-parasitic as well as an antidisease effect comes from the adoptive transfer of immune serum into malaria naïve individuals in the 1960s. Cohen and colleagues [44] purified γ-globulin from adult Gambians and transferred these preparations into Gambian children with high *P. falciparum* parasitemia. This treatment resolved fevers and reduced parasitemia in these children while nonimmune γ-globulin had no effect. Similar results were obtained in Nigeria in 1962 [45]. Both East African and Thai children could be treated with purified γ-globulin from immune West African adults, suggesting that either there are few regional differences in the distribution of parasite variants [46, 47] or that protective epitopes recognized by antibody are not strain specific.

Data from *in vitro* studies suggested that the protection seen in the passive transfer model was mediated by cytophilic antibodies that interact with monocytes. In an antibody-dependent cellular inhibition assay (ADCI), the protective IgG did not inhibit parasite growth and invasion *in vitro* when added alone to cultures, but did so when added in the presence of mononuclear cells from malaria naïve donors [48]. The protective and ADCI active sera were found to have high levels of the cytophilic antibodies IgG1 and IgG3 and relatively lower levels of IgG2 and IgM [49, 50]. IgG3 was associated with reduced frequency of malaria attacks in Senegal further strengthening the hypothesis that cytophilic IgG participates in protective mechanisms [51]. It has been suggested that the mechanism underlying ADCI involves the interaction of merozoites or Pf-iRBC with antibody and monocytes leading to the release of soluble mediator(s) responsible for parasite killing [52]. However, ADCI is not the only mechanism that could explain the massive reduction in

parasitemia observed in the passive antibody transfer experiments described above. In contrast to the observation by Bouharoun-Tayoun (1990), other studies have demonstrated that immune IgG can inhibit parasite growth *in vitro* in the absence of adherent cells [53, 54].

Studies in children living in malaria endemic areas have demonstrated the presence of antibodies to variant surface antigens (VSA) of various *P. falciparum* isolates in their sera [55, 56]. Both the antigenic and functional properties of VSA can be largely attributed to PfEMP-1 (reviewed in [57]). In an early longitudinal study in the Gambia [56], the titer of VSA antibodies was shown to be the only one of a series of immune assays that was associated with subsequent protection against disease. In a large longitudinal study of surface antigens of *P. falciparum*-infected erythrocytes from Kenyan children, malaria tended to be caused by parasite isolates expressing VSA variants corresponding to gaps in the repertoire of antibodies carried by the children before they became ill [58], an observation which has been confirmed in other studies [59]. Thus, immunity is associated with piecemeal acquisition of a repertoire of variant specific antibodies [56, 58], which may contribute to the slow acquisition of protective immunity to falciparum malaria. It now emerges that antibody responses to a variety of antigens are often short-lived and can only be detected in the presence of asymptomatic infection. Importantly, children harboring parasites and antibodies are protected from disease during the next malaria season whereas children with antibodies but no parasites tend to be more susceptible [60–62]. Because children will move between these groups – asymptomatically infected or free of parasites – within a given dry season or between dry seasons, these results indicate that asymptomatic infection induces protective, but short-lived, immune responses. The mechanisms underlying these protective immune responses are not clear.

31.5
Immune Recognition of iRBC

During asexual blood-stage malaria, the host is exposed to a considerable amount of foreign antigen in the circulation. After rupture of iRBC, when merozoites leave the RBC, additional debris is released into the bloodstream, which often coincides with the induction of fever. Both, the iRBC itself and debris after schizont rupture are recognized by pattern recognition receptors on monocyte/macrophages and DCs.

31.5.1
Toll-like Receptors

In the last year, TLR-mediated recognition of iRBC has been extensively investigated. The earliest report showed that mice lacking the adaptor molecule MyD88 failed to induce IL-12 and were protected from T-cell-mediated liver injury during the intra-erythocytic stages of the parasite [63]. Now evidence is accumulating that

P. falciparum GPI bind to TLR2 and TLR4 and hemozoin binds to TLR9, respectively. *P. falciparum* schizont lysate contained a ligand for TLR9, which induced IFN-α production in peripheral blood plasmacytoid DCs [64]. It has recently been demonstrated, that the unknown TLR9 ligand is hemozoin [65]. Hemozoin is the crystallized form of heme, a by-product resulting from digestion of hemoglobin by the parasite in RBC. When mature schizonts rupture, hemozoin, together with other debris, is released and rapidly taken up by neutrophils, monocyte/macrophages and DCs. *P. falciparum* hemozoin or synthetic β-hematin activated both $CD11c^{+}B220^{-}$ myeloid and $CD11c^{+}B220^{+}$ plasmacytoid DC derived from bone marrow from wildtype, TLR2, TLR4 and TLR7 knock-out mice but not from TLR9 knock-out mice. Crosslinking of TLR9 by natural or synthetic hemozoin resulted in the secretion of pro-inflammatory cytokines such as TNF-α and IL-6.

Likewise, Plasmodium GPI has long been suspected to induce inflammatory signals. It has recently been shown that GPI bound to TLR2 and to a lesser extent to TLR4 in both mouse and human macrophages and induced TNF-α secretion [66, 67]. Mouse macrophages also produced IL-12, IL-6 and NO in response to GPI when they were first primed with IFN-γ. It is perceivable that GPI will also activate human myeloid DC via TLR2 and TLR4. It was noted that free GPI is very quickly inactivated *in vivo* by phospholipases in serum and on cell surfaces. This may explain why activation of myeloid cells by Plasmodium GPI has long been suspected but very difficult to prove.

31.5.2 CD36

At least in human, binding of *P. falciparum* iRBC to CD36 expressed on monocyte/macrophages and dendritic cells plays an important role in non-opsonic clearance of iRBC. Non-opsonic phagocytosis of iRBC is directly correlated with the expression levels of CD36 [68]. In monocyte/macrophages, this process did not result in the production of TNF-α. In falciparum malaria, ligation of CD36 is a direct consequence of PfEMP-1 mediated adhesion of iRBC. Although mature iRBC are sequestered in post-capillary venules, a proportion of mature iRBC can be found in the spleen where iRBC might be removed by macrophages and DC in the perifollicular zone and in the marginal zone. Indeed, parasitemia in the spleen tends to be higher than in the peripheral circulation [69]. Thus, adhesion to CD36 expressed on endothelial cells favors multiplication of the parasite while adhesion to CD36 on monocyte/macrophages favors the removal of iRBC without the induction of inflammatory responses. Adhesion to CD36 is functionally conserved, in that most iRBC isolated from individuals with falciparum malaria bind to CD36. In addition, iRBC from children with mild malarial disease show a higher avidity for CD36 than isolates from children with severe malaria [70, 71]. Together, these observations suggested that adhesion of iRBC to CD36 has a role beyond sequestration in the regulation of immune responses to the parasite.

The role of CD36 adhesion in rodent malaria is less clear. *P. falciparum* iRBC bind to CD36 expressed on mouse macrophages [72]. However, although one re-

port showed that *P. chabaudi chabaudi* iRBC can bind to CD36, the evidence was indirect and awaits further investigation [73]. In mouse CD36 is expressed on monocyte/macrophages and on $CD8^+$ DC in the T-cell areas of lymph nodes and spleen but not on DC in the marginal zone.

31.5.3 Other Scavenger Receptors

In malaria, the role of scavenger receptors has not been extensively studied. SR-AI/II knock-out mice showed a similar course of parasitemia when infected with *P. chabaudi* iRBC to wildtype mice. However, blocking of non-SR-AI/II macrophage receptors with poly (I) resulted in earlier peak parasitemia *in vivo* and reduced phagocytosis of iRBC *in vitro*, suggesting that CD36, MARCO or macrosilain are involved in non-opsonic clearance of iRBC. In addition, blocking of the mannose receptor *in vitro* resulted in reduced phagocytosis [74].

31.5.4 Complement and Fc Receptors

Complement receptors and Fc receptors mediate opsonin-dependent phagocytosis of iRBC by monocyte/macrophages and DCs. In this respect, Fc receptors but not complement receptors are critical for the control of parasitemia in Plasmodium infected mice [75, 76]. Their role for DC maturation, conventional antigen presentation or cross-presentation has not been studied in Plasmodium infection in mice or man.

31.6 Dendritic Cells in Malaria

31.6.1 DCs in Human Malaria

Using monocyte-derived DCs, we have shown that intact *P. falciparum*-infected erythrocytes modulate DC maturation and function [77, 78]. Parasite-modulated DCs failed to upregulate the expression of MHC, co-stimulatory and adhesion molecules in response to stimulation with LPS, CD40-ligand, TNF-α or monocyte-conditioned medium ([77] and unpublished observations). Subsequently, both naïve and memory T-cells co-cultured with parasite-modulated DCs were functionally unresponsive with respect to proliferation and secretion of IL-2. Parasite-modulated DCs secreted IL-10 rather than IL-12, a cytokine which could inhibit T-cell activation. Both control DC and parasite-modulated DC produced TNF-α. These effects were in principle dependent on the ability of Pf-iRBC to adhere to CD36 and seemed to be mediated by PfEMP-1. Lysate of iRBC allowed normal dendritic cell maturation but affected antigen-processing, possibly due to the ingestion of hemo-

zoin [77]. In subsequent studies, similar modulation of DC had been shown with antibodies against CD36 [78] and MC-CIDR, a CD36 binding domain of one PfEMP-1 variant [Urban, unpublished observation].

A recent study by Skorokhod and colleagues showed that monocyte differentiation into DC is impaired in the presence of hemozoin [79]. In addition, DC co-cultured with hemozoin show an altered response to maturation signals. The impairment was accompanied by increased expression of the peroxisome proliferator-activated receptor-γ, upregulation of which is known to interfere with DC maturation [80, 81]. The effect on differentiation of monocytes appeared to be mediated by the same biochemical processes as hemozoin-induced changes in monocyte function. Hemozoin is not biochemically inert but reacts with membrane phospholipids and is transformed into hydroxy-polyunsaturated fatty acids, which cause membrane peroxidation [82, 83]. In addition, hemozoin catalysis induces the formation of prostaglandin PGE_2 and PGF_{2a}. While hydroxy-polyunsaturated fatty acids inhibit monocyte function such as phagocytosis, activation by inflammatory cytokines and generation of an oxidative burst, the release of PGE_2 and PGF_{2a} either by trophozoites or by monocytes, which have ingested pigment and/or trophozoites, alters T- and B-cell functions.

Plasmacytoid DC recently received attention, when a study by Pichyangkul *et al.* demonstrated that mature schizonts or lysate induced the expression of CD86 on and the secretion of IFN-α by plasmacytoid DCs *in vitro* [64]. These responses appear to be due to a soluble ligand of TLR9 in schizont lysate, most probably hemozoin. The same authors reported that the frequency of plasmacytoid DCs was reduced while the plasma levels of IFN-α were increased in Thai adults with both complicated and uncomplicated malaria.

Phenotypic analysis of DCs from patients who suffer from acute falciparum malaria can give some indication of DC function *in vivo*. In paraffin-fixed spleen sections from Vietnamese patients who died with falciparum malaria, we observed that myeloid DCs accumulate in the red pulp and in the marginal zone but not in the white pulp and T-cell areas. Furthermore, we noted a remarkable downregulation of HLA DR molecules on myeloid cells, including cordal macrophages and DCs, whereas HLA DR expression on sinusoidal lining cells was increased compared to controls [69]. This is in agreement with the observation that HLA DR expression is reduced on peripheral blood DCs in Kenyan children suffering from acute malaria [84].

31.6.2
DCs in Rodent Malaria

In contrast to human monocyte-derived DC, mouse bone-marrow derived DC incubated with the rodent parasite species *P. chabaudi chabaudi* readily increased the expression of co-stimulatory molecules and MHC molecules and produced inflammatory cytokines such as IL-12p70, TNF-α and IL-6. DC maturation in response to intact *P. chabaudi chabaudi* iRBC was further increased by the addition of LPS or TNF-α [85]. These observations were in agreement with a later study by Perry and colleagues, who purified $CD11c^+$ DC from spleen of mice infected with *P. yoelii* on

day 6 post-infection [86]. Splenic CD11c^{+} DC showed increased surface expression of CD40 and CD80 and induced IL-2 production in T cells. Leisewitz et al. reported that CD11c^{+} DC in the spleen of *P. chabaudi chabaudi* infected mice migrated from the marginal zone into the T-cell areas of the spleen as early as day 5 post-infection. In addition the surface expression of CD40 steadily increased from day 3 post-infection followed by CD54 and CD86 from day 7 onwards [87]. Together these results indicated that myeloid DCs are readily activated, migrate and secrete inflammatory cytokines when exposed to iRBC of rodent *Plasmodium* species either *in vitro* or *in vivo*.

However, other authors showed that bone-marrow derived DC failed to mature and to activate T-cells when co-cultured with *P. chabaudi chabaudi* or *P. yoelii* iRBC. In one study, these DC retained immunostimulatory capacity because mice injected with iRBC-loaded DC were protected from death when challenged with an otherwise lethal dose of *P. yoelii*. Nevertheless, these mice still experienced high levels of parasitemia [88]. It seems possible that in these experiments, antigen was transferred from injected, iRBC-loaded DCs to resident DCs [89]. Such a scenario might explain why apparently modulated DC allowed protection from death while affecting parasitemia only marginally: resident DC were not exposed to intact iRBC but to partially broken down antigen.

Ocana-Morgner and colleagues also observed that *P. yoelii* infected iRBC inhibited DC maturation *in vitro* in response to LPS and *in vivo*. In addition, the ratio of IL12 and IL10 secreted by DC was reversed. Subsequently, Ocana-Morgner showed, that blood-stage malaria inhibited the CD8^{+} T-cell response to the liver-stage of the parasite. The mechanisms are not completely understood but most probably due to soluble factors secreted by DC [90].

31.7 Synopsis

Despite apparent heterogenous reports on DC function in rodent malaria, common patterns can be established (Table 31.1). *P. chabaudi* iRBC seem to be less prone to modulate DC function than *P. yoelii* iRBC. These differences could be due to parasite-encoded surface proteins involved in the interaction of iRBC with DC. Variant surface antigens of rodent Plasmodium species are now under active investigation, although a link between cytoadhesion and antigenic variation has not been established. In addition, the genetic background of mouse strains used in these experiments directly influences the kinetics and cellular composition of the immune response to a given parasite strain. This restriction may well have an effect on the responsiveness of DC to activation and determine the cytokine environment in which it occurs. In addition, DC modulation seems to be dependent on parasite inoculation rates or DC:iRBC ratio used in different experiments. Low ratios of DC to iRBC *in vitro* or low parasitemia *in vivo* seem to favor DC maturation, whereas high ratios *in vitro* or parasitemia *in vivo* appears to result in the inhibition of DC function. Whether this is due to active inhibition of DC function or a conse-

Tab. 31.1 DC function in rodent malaria.

Parasite	*Assay*	*DC*	*Dose*[a]	*Para*[b]	*DC:iRBC*[c]	*Outcome*	*Ref.*
P. chabaudi	*in vitro*	BM-DC			1:30	• mature iRBC induce surface expression of co-stimulatory molecules and secretion of cytokines	85
P. chabaudi	*in vivo*	CD11c$^+$	10^5	>5%		• migration of CD11c$^+$ DC into the T-cell zones on day 5	87
				> 5%		• upregulation of CD40 on day 5	
				13%		• upregulation of CD54, CD86 on day 7	
P. chabaudi / P. yoelii	*in vitro*	BM-DC			1:10	• no upregulation of co-stimulatory molecules in response to LPS	88
	in vitro/ in vivo	BM-DC	10^6		1:10	• transfer of iRBC loaded BM-DC into mice protected from death but not parasitemia after subsequent challenge with a lethal dose of iRBC	
P. yoelii	*ex vivo*	CD11c$^+$		13%		• DC upregulated surface expression of co-stimulatory cytokines • DC induced activation and cytokine secretion in T cells	86
P. yoelii	*in vitro/ in vivo*	BM-DC CD11c$^+$	4 x 10^6		1:100	• iRBC inhibited increase in surface expression of co-stimulatory molecules and reversed ratio of IL12:IL10 secretion	90
	in vivo		4 x 10^6			• Inhibition of liver-stage specific CD8$^+$ T cells due to soluble factor secreted by DC	

[a] number of iRBC used for infection of mice where known
[b] parasitemia at the time of assay where known
[c] ration of DC to iRBC used in *in vitro* assays

quence of the resolution of inflammation will have to be established in more detailed studies.

In principle a similar effect of parasitemia on DC function should apply to falciparum malaria in humans. It may even be more pronounced, because CD36-mediated modulation of myeloid DC is a contact-dependent process. It suggests that early on during infection, plasmacytoid DC could be induced to secrete IFNα through engagement of TLR9 by hemozoin. In parallel, myeloid DC might be activated via ligation of TLR2 and 4 or cytokines such as IFN-α and IFN-γ secreted by plasmacytoid DCs, NK cells, NKT cells, secrete IL12 and other cytokines resulting in the activation of $CD8^+$ and $CD4^+$ T-cell responses. With increasing parasitemia, more and more myeloid DC in the spleen might be modulated either by direct interaction with iRBC or through ingestion of increasing amounts of hemozoin (Fig. 31.1). Modulation of myeloid DCs at best might prevent the induction of additional T-cell responses or at worst alter already existing T-cell responses or induce regulatory T cells. Given that severe malarial disease, at least in part, is mediated by inflammatory immune responses, modulation of DC might prove to be beneficial to the host rather than exuberating disease. Clearly, detailed analysis not only of DC function during the course of Plasmodium infection but also the consequences for T- and B-cell responses are required both in human falciparum malaria as well as in rodent models of infection.

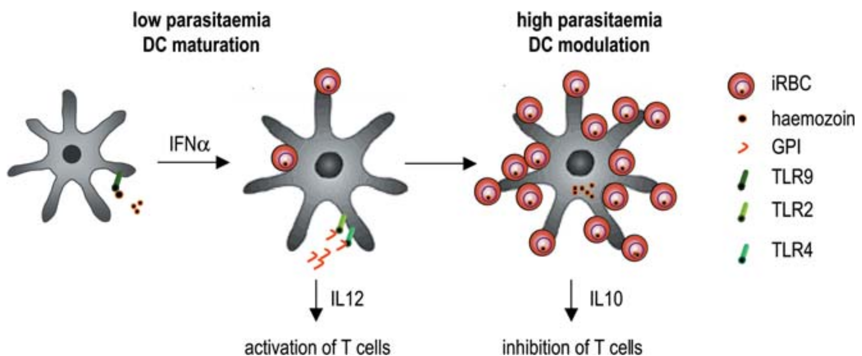

Fig. 31.1 Simplified diagram on the effect of parasitemia on DC function. Early on during infection, engagement of TLR9 by hemozoin results induces plasmacytoid DC to secrete IFNα and engagement of TLR2 and TLR4 by GPI induces myeloid DC to secrete IL12. With increasing parasitemia, more and more myeloid DC in the spleen might be modulated either directly through interaction with iRBC or through ingestion of increasing amounts of hemozoin and secrete IL10.

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32 Dendritic Cells in Leishmaniasis: Regulators of Immunity and Tools for New Immune Intervention Strategies

Heidrun Moll

32.1 Introduction

Leishmaniasis is considered a tropical affliction that is included in the World Health Organization/Tropical Disease Research list of the six most important diseases. Disability-adjusted life years (DALYs) lost due to leishmaniasis are close to 2.4 million, the world-wide incidence is estimated to be 12–15 million cases, and a population of 350 million is at risk [1]. There has been a sharp increase in the number of recorded cases in recent years. For example, the current epidemic in Afghanistan affects hundreds of thousands of people. Leishmaniasis is endemic in Central and South America, India and the Middle East, but also in southern European countries, such as Portugal, Spain, Greece and Italy. There is an increased interest in leishmaniasis in industrialized countries, due to the importance of travel medicine and the rising incidence of human immunodeficiency virus (HIV) and *Leishmania* co-infections. Especially in south-western Europe, the mutual reinforcement associated with HIV and *Leishmania* co-infection is considered a real threat: leishmaniasis accelerates the onset of AIDS and shortens the life expectancy of HIV-infected people, and infection with HIV can increase the risk of leishmaniasis by 100–1000 times in endemic areas [1, 2].

In addition to these clinical aspects, the interest in leishmaniasis is based on its importance as a model to define the factors controlling the development of polarized T helper (Th)1 and Th2 cell responses. In fact, the relevance of the Th1/Th2 cell balance *in vivo* to the outcome of a disease was first documented in the model of murine leishmaniasis with *Leishmania major* [3, 4]. This experimental system has provided a wealth of information on the immunological mechanisms leading to the restriction or facilitation of pathogen growth, with implications not only for infectious diseases but also for general aspects of immunoregulation.

Leishmaniasis is caused by protozoa of the genus *Leishmania* which are transmitted by sand flies. The parasites alternate between the flagellated promastigote form in the insect vector and the obligatory intracellular amastigote form in the mammalian host. They induce a group of diseases (Table 32.1) that vary in severity

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Tab. 32.1 Spectrum of diseases induced by *Leishmania* parasites.

Disease	***CL***	***DCL***	***MCL***	***VL***
Major parasite species	*L. major* *L. tropica* *L. mexicana* *L. aethiopica*	*L. mexicana* *L. aethiopica*	*L. amazonensis* *L. brasiliensis*	*L. donovani* *L. infantum* *L. chagasi*
Major clinical symptoms	localized skin lesions	disseminating skin lesions	destruction of oro-nasal and pharyngeal mucosal tissue	fever, weight loss, malaise, hepato-splenomegaly, anemia
Disease outcome	self-healing	chronic, progressive	chronic, progressive	progressive, may be fatal if untreated

CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis.

from the self-healing cutaneous leishmaniasis (oriental sore), characterized by a localized skin lesion at the site of the sand fly's bite, to potentially fatal visceral disease (kala-azar), in which the parasites disseminate and invade lymph nodes, spleen, liver and bone marrow [5]. The clinical manifestation depends not only on the parasite species, but also involves the genetic basis of the host's ability to mount an effective cell-mediated immune response, thus resembling the situation in leprosy. In contrast to viral and bacterial infections, no vaccines are available to protect humans from parasitic diseases including leishmaniasis and, therefore, control measures rely exclusively on chemotherapy. The current treatments for leishmaniasis are unsatisfactory due to their toxic side effects, expense and the increasing problems with drug resistance. Thus, there is an urgent need to develop novel strategies for the prevention and treatment of leishmaniasis and other parasite infections. A thorough understanding of the complex immune mechanisms resulting in resistance or pathology is a prerequisite for the elaboration of new approaches to be used for vaccination and immunotherapy.

32.2 Mechanisms Mediating Resistance or Susceptibility to Leishmaniasis

Leishmania parasites that are pathogenic to man also infect mice, and the spectrum of diseases seen in humans can be mimicked by infection of different strains of inbred mice with *L. major*, a cause of human cutaneous leishmaniasis. Murine infection with *L. major* parasites is perhaps the disease model that has been used most widely to study the cell populations and cytokines involved in host resistance or susceptibility to a microbial pathogen. A plethora of data demonstrated that the course of disease depends on the type of the host's immune response (Fig. 32.1).

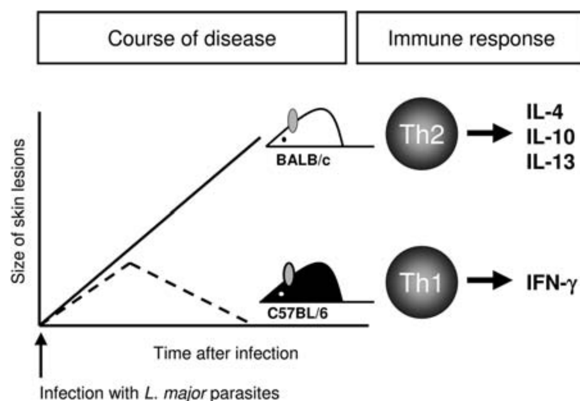

Fig. 32.1 Th-cell polarization correlates with resistance or susceptibility to experimental leishmaniasis. After infection with *L. major*, a cause of human cutaneous leishmaniasis, mice of the inbred strain C57BL/6 develop skin lesions that resolve spontaneously and lead to the generation of life-long immunity to re-infection, whereas BALB/c mice develop progressive lesions with systemic disease and fatal outcome. The ability of C57BL/6 mice to control *L. major* infection is associated with the development of Th1 cells producing IFN-γ. In contrast, the development of Th2 cells secreting IL-4, IL-10 and IL-13 predominates in susceptible BALB/c mice.

Protective immunity is associated with the development of a Th1 response, while a Th2 cytokine pattern prevails in mice that succumb to infection [6]. Interleukin (IL) 12 is considered to have a central regulatory function in directing the development of protective Th1 cells. These findings were paradigmatic for many infectious diseases.

32.2.1 The Role of T Helper Cell Subsets

Experimental infection of mice with *L. major* is an excellent model to explore the development and functions of Th-cell subsets *in vivo*. Most strains of inbred mice, such as C57BL/6 and C3H, are able to restrain the disease, with skin lesions resolving spontaneously. In contrast, mice of some other strains, such as BALB/c, are highly susceptible to infection, with parasites disseminating to the viscera and fatal outcome. The healing of lesions in resistant mice is linked with the induction of Th1 cells secreting interferon-γ (IFN-γ). This cytokine stimulates the parasiticidal activity of macrophages via expression of inducible nitric oxide synthase (iNOS) and formation of reactive nitrogen metabolites, resulting in the killing of the intracellular *Leishmania*. The inability of BALB/c mice to control *L. major* infection is associated with early IL-4 production by $CD4^+$ T cells and the development of a sustained Th2 response. This Th2 dominance suppresses the development of Th1 cells in susceptible mice. However, it is important to note that an early, although transient, production of IL-4 can also be detected in *L. major*-infected mice of re-

sistant strains [7–9]. Thus, a critical factor determining resistance is the ability to redirect the early IL-4 response following infection to a protective Th1 pathway.

The development of a Th1 response and resistance to leishmaniasis is driven by IL-12. Depletion of this cytokine by genetic means or antibody neutralization abrogates the ability of resistant mice to clear the infection, while treatment of susceptible mice with IL-12 induces protection [10–12]. These data show that IL-12 is necessary to redirect the early Th2 response. The essential role of IL-12 in resistance to leishmaniasis is not confined to its involvement in the initiation of a protective Th1-mediated response, but IL-12 is also required for the maintenance of immunity to *Leishmania* [13, 14]. When IL-12-deficient mice were treated with recombinant IL-12 only transiently during the initial stage of infection, they eventually developed progressive disease that was associated with a loss of the Th1 profile and the development of a Th2 cytokine pattern [13]. It has been suggested that the importance of IL-12 in maintaining an established Th1 response may be based on its ability to (1) induce optimal proliferation and IFN-γ production by Th1 cells, (2) ensure Th1 cell survival by preventing apoptotic cell death and (3) prevent Th2 cell development by replenishing the pool of Th1 cells from uncommitted Th cells or central memory T cells [15].

Another susceptibility factor for *L. major* infection is IL-13 [16]. Mice deficient for both IL-4 and IL-13 are more resistant than either single knock-out strain, suggesting that IL-13 effectively cooperates with IL-4 to promote the development of Th2 cells in *L. major*-susceptible mice. In addition, IL-10 is involved in disease exacerbation and the instruction of Th2 cells. Two sources of IL-10 may be relevant in leishmaniasis, macrophages and $CD4^+$ T cells. IL-10 can be a component of the Th2 cytokine profile, but it is also produced by other $CD4^+$ subsets, in particular $CD4^+$ $CD25^+$ regulatory T cells (Treg cells).

32.2.2
The Role of Regulatory T Cells

Treg cells are specialized subsets of $CD4^+$ T cells that negatively regulate various cell-mediated immune functions. They are characterized by the expression of high levels of IL-10 and transforming growth factor-β, cytokines involved in the downregulation of both macrophage effector functions and Th1 differentiation. The importance of Treg cells in leishmaniasis was demonstrated by the finding that these cells represent a large proportion of $CD4^+$ T cells in chronic and in healed lesions [17, 18]. They are activated during infection to suppress *Leishmania*-specific Th1 immunity in resistant mice, thereby preventing sterile cure, and may be important to limit the tissue damage associated with sustained inflammatory immune responses. This effect of Treg cells was shown to depend on their ability to produce IL-10 [17, 19]. In conclusion, although multiple pathways control resistance or susceptibility to leishmaniasis, it is clear that the Th1/Th2 balance regulates the disease outcome *in vivo*.

32.3 Dendritic Cell Interaction with *Leishmania* Parasites

Leishmania parasites are transmitted by the bite of infected sand flies depositing the infectious load in the skin of the mammalian host. At this site, a number of distinct potential phagocytes and antigen-presenting cells (APC) are present, including epidermal Langerhans cells, dermal dendritic cells (DC) and dermal macrophages. The early interaction of the pathogen with host cells is likely to be critical for the course of infection. Therefore, it is important to understand the mechanisms facilitating or counteracting the establishment of parasite infection.

32.3.1 Parasite Uptake by Dendritic Cells

Leishmania promastigotes attach to specialized surface receptors on mononuclear phagocytes and are rapidly taken up by phagocytosis. The bulk of parasites is harbored by macrophages, but Langerhans cells and dermal DC, which are known to monitor peripheral tissues for invading pathogens [20, 21], also ingest the microorganisms. DC express a variety of receptors for pathogen-associated molecular patterns, and at least two of them are involved in the uptake of *Leishmania* parasites. The phagocytosis of *L. major* by *ex vivo*-derived murine Langerhans cells was demonstrated to be mediated by CR3, the receptor for complement component C3bi [22]. More recently, using immature human DC, it was shown that a C-type lectin, the DC-specific intercellular adhesion molecule (ICAM)-3grabbing nonintegrin (DC-SIGN, CD209), is also involved in the uptake of *Leishmania* parasites by DC [23]. DC-SIGN appears to discriminate among *Leishmania* species because it bound *L. pifanoi*, a cause of New World cutaneous leishmaniasis, as well as *L. infantum* and *L. donovani*, causing visceral leishmaniasis, with high avidity, while it interacted only poorly with *L. major*, a species responsible for cutaneous pathology in the Old World [24]. Interestingly, opsonized *L. infantum* parasites were found to exhibit a much lower capacity to bind to DC-SIGN [24], raising questions about its role in the interaction of *Leishmania* and DC *in vivo* because in physiological conditions the majority of parasites are likely to be opsonized by serum components, such as antibodies and complement factors. It has been shown that DC are able to phagocytose *Leishmania* regardless of whether they had previously been opsonized [25].

Leishmania glycosylinositolphospholipids, high mannose-containing molecules abundantly expressed on the surface of both promastigotes and amastigotes, appear not to be involved in parasite binding to DC via DC-SIGN [23]. In contrast, *L. mexicana* lipophosphoglycan (LPG), the mannose-capped major surface glycoconjugate of promastigotes, was proposed as a *Leishmania* ligand for DC-SIGN [26]. However, the binding of *L. donovani*, *L. infantum* and *L. pifanoi* to DC-SIGN was shown to be independent of LPG [24] and, because LPG-defective parasite strains bound DC-SIGN with even higher avidity than their wild-type counterparts, it was

suggested that LPG may in fact mask other DC-SIGN ligands on the *Leishmania* surface membrane [24]. Thus, it remains to be clarified which parasite molecules are involved in the attachment to DC receptors.

The ability to ingest *Leishmania* parasites of different life cycle stages, amastigotes versus promastigotes, seems to depend on the origin of DC. While Langerhans cells and Langerhans cell-like fetal skin-derived DC have been reported to take up amastigotes, but not promastigotes [22, 27], DC isolated from spleen and DC generated from bone marrow or peripheral blood precursor cells by *in vitro* culture in the presence of IL-4 and/or granulocyte-macrophage colony-stimulating factor were shown to internalize promastigotes and amastigotes [25, 28–30]. However, regardless of the type of DC and the infective stage of *Leishmania*, it has generally been found that the uptake of parasites by DC is not as efficient as that seen with macrophages. The percentage of parasitized DC is significantly lower and each infected DC usually harbors only 1 to 2 microorganisms, whereas macrophages may contain a tenfold number of parasites. This is a notable finding strongly suggesting that macrophages and DC play distinct roles in leishmaniasis. In contrast to macrophages, the uptake of parasites by DC is not aimed at avid scavenging and clearance of the pathogen but at antigen processing and presentation to T cells (see Section 32.4)

32.3.2 Subcellular Location of *Leishmania* Parasites in Dendritic Cells

Inside host cells, the parasites initially reside in phagosomes which subsequently fuse with lysosomes to form phagolysosomes. While this process of endosome maturation is well characterized in parasitized macrophages [31], there is little information about the fate of phagosomes in DC harboring *Leishmania* or other intracellular pathogens. A distinct feature of DC is the variation of their properties with the different stages of their life span. DC have highly specialized endocytic structures, which are regulated upon maturation. DC in nonlymphoid tissues, such as the skin and the gut, are immature cells which can phagocytose and process particles, but are only weak stimulators of T-cell immune responses. The differentiation of DC is triggered by exposure to pathogens or inflammatory cytokines and is accompanied by the loss of endocytic activity and a marked upregulation of the expression of molecules involved in antigen presentation.

Analysis of the *Leishmania*-containing parasitophorous vacuole (PV) in DC by confocal immunofluorescence microscopy revealed that the parasites reside in an acidic compartment containing major histocompatibility complex (MHC) class II and H-2M molecules, characteristics of the MIIC [32], macrosialin (CD68) and the lysosome-associated membrane proteins (Lamp) 1 and 2, which are markers of late endosomes and lysosomes (Fig. 32.2) [25, 29, 33]. While these features are similar to those of infected macrophages, the finding that the PV in DC, in contrast to macrophages, express low levels of rab7p, a molecule involved in the fusion of late endosomes with lysosomes, and cysteine and aspartyl proteases, like cathepsin B, H, L and D, support the idea that PV biogenesis is different in DC and macrophag-

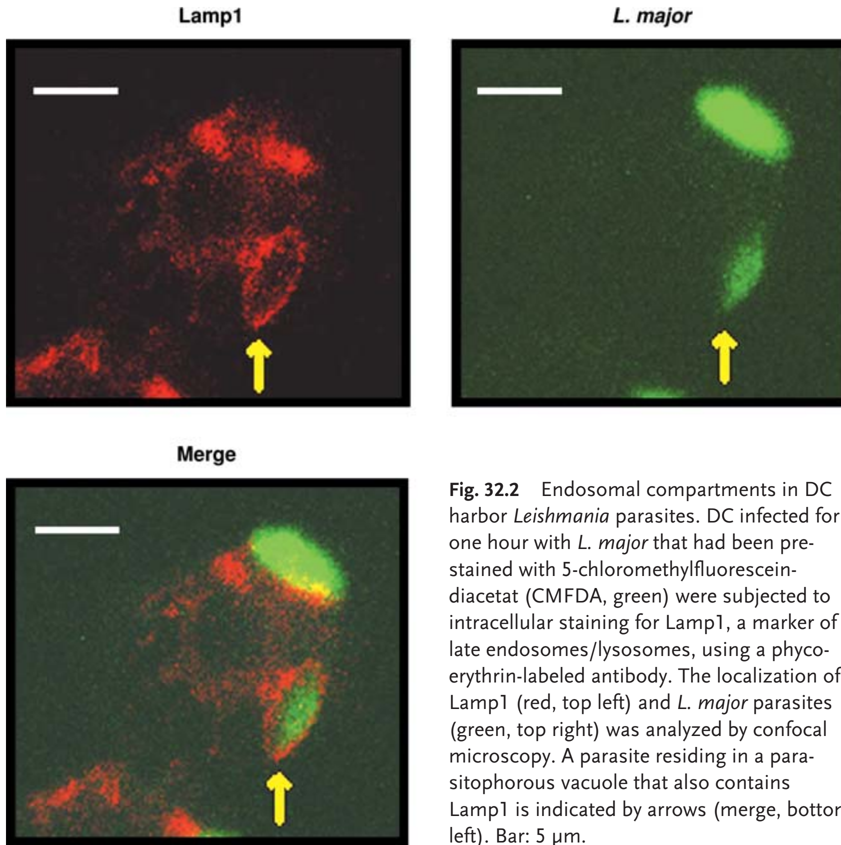

Fig. 32.2 Endosomal compartments in DC harbor *Leishmania* parasites. DC infected for one hour with *L. major* that had been prestained with 5-chloromethylfluorescein-diacetat (CMFDA, green) were subjected to intracellular staining for Lamp1, a marker of late endosomes/lysosomes, using a phycoerythrin-labeled antibody. The localization of Lamp1 (red, top left) and *L. major* parasites (green, top right) was analyzed by confocal microscopy. A parasite residing in a parasitophorous vacuole that also contains Lamp1 is indicated by arrows (merge, bottom left). Bar: 5 μm.

es [25]. This may have consequences for the parasite antigen presentation functions of DC.

32.3.3
Dendritic Cell Subsets Involved in the Uptake of *Leishmania*

DC can be separated into subsets which differ in phenotype, function and localization [34]. Three distinct DC subpopulations have been identified in mouse spleen, as determined by the expression of CD4 and CD8α molecules on their surfaces, and the existence of five DC subpopulations was documented for skin-draining lymph nodes, with the two additional subsets originating from the skin (Langerhans cells and dermal DC). Each of these subsets was shown to exhibit a different pattern of cytokine secretion [35]. Interestingly, the three subpopulations of murine splenic DC can be ranked on the basis of their ability to internalize *L. major* parasites [29]. $CD4^+$ $CD8^-$ DC have the highest infection rate, followed by $CD4^-$ $CD8^-$ DC. The $CD4^-$ $CD8^+$ subpopulation of DC showed the lowest level of parasite uptake. This hierarchy in the phagocytosis activity of the different DC populations correlated

with the levels of their CR3 expression [29], supporting the previous suggestion that this complement receptor is critical for the uptake of *Leishmania* by DC [22].

32.3.4
Dendritic Cells in *Leishmania*-infected Tissues

As summarized above, a number of studies demonstrated that DC can phagocytose *Leishmania in vitro*. Moreover, parasite antigen-containing DC can also be detected *in vivo* in tissues of *Leishmania*-infected mice and humans [22, 36–38]. Inadequate epidermal homing of Langerhans cells has been described to lead to tissue damage in patients with chronic cutaneous leishmaniasis [39, 40].

In mouse ear skin infected with *L. major*, immunohistologic staining revealed distinct clusters of $CD205^+$ Langerhans cells in the parasite-containing dermal infiltrate. These cells constituted less than 1% of the mononuclear cells in the infiltrate and some of them expressed parasite antigen, as demonstrated by double labeling [22]. Langerhans cells in the epidermis were found not to be parasitized. In contrast to the primary lesion, in which macrophages and granulocytes appear as the mainly infected cells, $CD11c^+$ DC were shown to be the most frequently infected cell population in the draining lymph nodes during the peak of *L. major* infection in both resistant C57BL/6 and susceptible BALB/c mice [37]. In lymph nodes of infected BALB/c mice, DC presented high parasite loads, suggesting that *in vivo* infection might affect the physiology of DC more strongly, possibly due to the duration of infection and/or the presence of environmental factors. In addition, $CD11c^+$ multinucleated giant cells harboring a dramatic accumulation of parasites were frequently observed [37].

The characterization of DC subpopulations purified from the draining lymph nodes of *L. major*-infected mice revealed a larger number of plasmacytoid DC in susceptible BALB/c mice than in resistant C57BL/6 mice [41]. Interestingly, when the parasite load in the different DC subpopulations of the lymph nodes draining the lesion was examined in the course of infection, the frequency of cells carrying live parasites was similar for all the subpopulations, but the plasmacytoid DC in resistant mice continued to harbor parasites for the longest period. The possible consequences of these differences remain to be elucidated but it is conceivable that the high levels of type I IFN (IFNα/β) expression known to be associated with plasmacytoid DC [42] may influence the quality of the immune response. In contrast to classical DC subsets, plasmacytoid DC lack the ability to stimulate naïve T cells, but can effectively promote the differentiation of antigen-experienced unpolarized T cells into Th1 cells [43].

32.4
Dendritic Cell Migration and Induction of a *Leishmania*-specific Immune Response

The movement of DC precursors into sites of microbial infection in peripheral tissues is regulated by the sequential involvement of chemokines, such as monocyte

chemotactic protein (MCP) and macrophage inflammatory protein (MIP)-3α/CCL20, binding to the chemokine receptors CCR2 and CCR6, respectively, on the DC surface. Immature DC also respond to various inducible chemokines that are released by macrophages upon activation by microbial products, such as CCL3 (MIP-1α), CCL5 (RANTES) and CXCL8 (IL-8), the ligands of the receptors CCR1, CCR5 and CXCR1 expressed by these DC, and thus accumulate at the site of infection [44]. In the skin, Langerhans cells represent immature DC with a high endocytic activity that are able to take up microbes and/or microbial antigens for degradation and peptide loading onto MHC molecules [45]. The recognition of microbial signals induces the maturation of DC, a process that is accompanied by their efflux from the skin and migration to the T-cell areas of draining lymph nodes [46]. During maturation, the endocytic capacity of DC is downregulated, while their expression of MHC class II as well as co-stimulatory and adhesion molecules, such as CD80, CD86 and CD40, is strongly upregulated [20]. Thus, they acquire the distinct ability to trigger a primary T-cell response. The expression of the inflammatory chemokine receptors CCR1, CCR2, CCR5 and CCR6 decreases in the course of DC maturation and, therefore, the responsiveness to the corresponding ligands is reduced. At the same time, DC gain surface expression of the chemokine receptor CCR7 and responsiveness to its ligands CCL19 (MIP-3β) and CCL21 (secondary lymphoid tissue chemokine) that are produced in the T-cell areas of lymph nodes. CCR7 is a key receptor for the convergence of antigen-loaded mature DC and responder T cells, as it also mediates the homing of activated T cells to the lymph nodes [47].

32.4.1
The Role of Chemokines and Chemokine Receptors Expressed by Dendritic Cells

Leishmania parasites have been shown to selectively modulate the expression of chemokines and chemokine receptors by DC in a species-dependent manner. DC from mice infected with *L. donovani* were reported to have reduced expression of CCR7 and a decreased responsiveness to CCR7 ligands [48]. This is associated with a failure of DC from chronically infected mice to migrate from the marginal zone to the periarteriolar region of the spleen. Treatment with CCR7-expressing DC provided protection against parasite growth, demonstrating that defective CCR7-mediated DC migration plays a major role in the pathogenesis of visceral leishmaniasis. In contrast, interaction of DC with *L. major* was shown to enhance the level of CCR7 expression and the DC response to its ligand CCL21 [49]. Expression of the chemokine receptors CCR2 and CCR5, however, and the responsiveness to the respective ligands CCL2 (MCP-1) and CCL3 (MIP-1α) were downregulated by DC exposure to *L. major* [49]. These alterations in chemokine receptor expression induced by *L. major* were observed with DC from resistant and susceptible mice, indicating that the differential ability of these mice to control cutaneous leishmaniasis is not due to host-dependent effects of the parasite on chemokine receptors directing DC migration. Notably, *L. major* elicited expression of the chemokine CXCL10 (IFN-inducible protein, IP-10) only in DC from resistant mice. This obser-

vation extended earlier studies [50, 51], which showed that *L. major* infection upregulates CXCL10 expression in the draining lymph nodes of resistant, but not susceptible mice, by demonstrating that CXCL10 derived from *L. major*-stimulated DC may account for the higher level of this chemokine in resistant mice. CXCL10 recruits and activates natural killer (NK) cells, a component of the innate immune system, that have been implicated in the development of resistance to leishmaniasis [52].

The importance of chemokine receptors for DC functions and the course of *L. major* infection was also tested with mice lacking CCR2 or CCR5 [53]. This study showed that deficiency of CCR2, a receptor for CCL2 (MCP-1), but not CCR5, or its ligand CCL3 (MIP-1α), leads to distinct defects in DC biology. While the density of Langerhans cells in the epidermis of CCR2-deficient mice and their ability to move to the dermis was normal, their migration to the draining lymph nodes was strongly impaired. CCR2 knock-out mice had decreased numbers of DC in the spleen and a block in the *L. major* infection-induced relocalization of DC from the marginal zone to the T-cell areas of the spleen. CCR2-deficient mice with a *L. major*-resistant genetic background were susceptible to disease and developed a Th2-dominated cytokine profile. These findings demonstrated that CCR2 plays an important role in DC migration and localization, with critical impact on the development of a protective immune response to *Leishmania*.

32.4.2
Transport and Presentation of *L. major* Antigen by Dendritic Cells

After intradermal infection of mice with *L. major*, a significant change in the distribution of Langerhans cells was observed [22]. A considerable loss of $CD205^+$ Langerhans cells in the segment of the epidermis overlying the parasite-containing infiltrate was concomitant with the appearance of those cells in the dermal layer of the lesion, some of which contained *L. major* antigen. These observations indicated that Langerhans cells migrate from the epidermis to the site of infection in the dermis for uptake of *L. major* parasites and/or parasite antigen. Moreover, the cells were demonstrated to migrate to the draining lymph node within 24 to 48 h [36], using double labeling immunohistochemistry as well as *in vivo* tracking of labeled Langerhans cells that had been infected with *L. major in vitro* and were reinjected into the skin. Such a translocation was not observed with infected macrophages under equivalent conditions. The migratory DC in the lymph nodes expressed *L. major* antigen and were able to stimulate resting T cells to mount a *L. major*-specific immune response *in vivo*. These findings suggested that Langerhans cells transport *L. major* parasites and/or parasite antigen from the skin to the regional lymph nodes for initiation of the specific T-cell immune response [54, 55].

The role of DC subpopulations in parasite dissemination from the infected skin to the draining lymph node was recently examined in detail [41]. Using ear skin explants from *L. major*-infected resistant or susceptible mice, it was shown that the majority of DC emigrating from the skin were Langerhans cells (20% on average),

whereas only a small population of migratory dermal DC was observed (less than 5%). However, no parasites could be detected in DC emigrating from infected skin explants, while macrophages harbored significant numbers. Moreover, viable parasites were not detectable in DC subpopulations purified by flow cytometry from draining lymph nodes until 3 weeks after infection. Antigen presentation functions were not examined in this study. The authors concluded that macrophages but not DC are the vehicle that ferries parasites from skin to lymph nodes and that DC take up parasites from infected macrophages in the lymph nodes. This seems to be at odds with the earlier findings described above [22, 36]. However, the following points need to be considered. (1) Fundamentally different experimental approaches were used in these studies. The migratory behavior of DC in skin explants is likely to differ from that of DC *in vivo* because chemokines direct them from skin to lymph nodes. Furthermore, the methods used for detection of *L. major*-loaded DC in the lymph nodes (immunocytochemistry of tissue sections versus DC isolation by flow cytometry) may differ in sensitivity; *in vivo* tracking of labeled DC revealed that only a very small proportion of the cells (0.1–0.5%) migrate to the draining lymph node [36]. (2) Different time points were used for the analysis of DC in the draining lymph nodes. While no DC containing live parasites were detected at day 8, the earliest time point analyzed in the one study [41], small numbers of DC expressing *L. major* antigen could be demonstrated in the early phase of infection (1–4 days) in the other study [36]. (3) Finally, it is important to note that DC do not need to carry live parasites to fulfill their prime task, i.e. the initiation of a specific T cell-mediated immune response. It has been shown that DC, but not macrophages, pulsed with parasites or cell-free *L. major* culture filtrates are able to stimulate a primary T-cell response [28]. This idea is supported by the finding that two waves of *L. major* antigen-containing $CD205^+$ DC appear in the draining lymph nodes. The DC of the first wave become detectable at 8 h after infection but do not harbor intact parasites, while DC carrying live parasites were observed in the second wave at 24–48 h after infection [56]. The DC of the first wave were shown to prime parasite-specific $CD4^+$ T cells.

Using a mouse model in which MHC class II expression is restricted to $CD11b^+$ and $CD8\alpha^+$ DC, it has recently been demonstrated that antigen presentation by these DC subsets is sufficient for T-cell priming, induction of Th1 differentiation and control of subcutaneous *L. major* infection [57]. Antigen presentation by macrophages was not required. In these mice, plasmacytoid DC and Langerhans cells also lack MHC class II and, thus, were not involved in the activation of parasite-specific Th cells. It will be important to assess the requirement for MHC class II expression by Langerhans cells in intradermal infection models. In another recent report, also using subcutaneous infection with *L. major*, it has been suggested that dermal $CD8\alpha^-$ Langerin-negative DC transport *L. major* antigen to the lymph nodes and induce a secondary proliferative response of $CD4^+$ T cells [58]. However, their ability to stimulate a primary T-cell response to *L. major* and Th1-mediated control of cutaneous disease was not examined in this study.

32.4.3
Parasite Persistence in Immune Hosts

After clinical cure of cutaneous leishmaniasis in resistant mice, life-long immunity is maintained but small numbers of parasites persist, preferentially in the lymph nodes draining the prior skin lesion, even in the face of a competent immune response [59]. *L. major* parasites were found to be sequestered in macrophages, fibroblasts and DC [60–62]. The sustained expression of iNOS, IL-12 and IFN-γ is crucial for the control of latent infection, because impairment of either of these responses in cured mice has been shown to cause clinical recrudescence of the disease [14, 19, 62]. Sterile cure is achieved in IL-10-deficient mice after healing and by treatment with anti-IL-10 receptor antibodies [19], but the observation that these mice are no longer immune to re-infection [17] suggests that antigen persistence is required for the maintenance of protective immunity. Notably, the examination of latently infected host cells isolated from draining lymph nodes of cured mice demonstrated that only DC, but not macrophages, are able to present endogenous *L. major* antigen to specific T cells *in vitro* [61]. Thus, DC may contribute to the sustained stimulation of effector memory T cells and long-term control of the parasite. This extraordinary efficiency in antigen presentation by DC may be explained by the highly increased stability of MHC class II molecules loaded with immunogenic parasite peptides [33].

32.5
Regulation of the *Leishmania*-specific Immune Response by Dendritic Cells

Microbial structures are recognized by Toll-like receptors (TLR) expressed on the surface of DC. TLR signaling induces the maturation of DC and stimulates the release of inflammatory cytokines, thus enabling DC to prime T cells and shape the developing cell-mediated immune response. In this way, TLR are involved in the link between innate and adaptive immunity [63, 64]. The different TLR possess conserved cytoplasmic domains and use common intracellular signaling pathways that involve recruitment of the cytoplasmic adaptor molecule MyD88, activation of serine/threonine kinases of the IRAK family, and finally degradation of IκB and translocation of NF-κB to the nucleus. A MyD88-independent pathway has been shown to be responsible for the induction of IFN-β and IFN-inducible genes.

Systemic administration of *Leishmania* parasites (*L. brasiliensis, L. donovani, L. major* or *L. mexicana*) was shown to induce full DC maturation *in vivo*, as hallmarked by DC migration from marginal zones to T-cell areas of the spleen and upregulation of MHC class II and the co-stimulatory molecules CD40, CD80 and CD86 [65]. Comparable maturation processes were observed for DC from genetically susceptible and resistant mice, indicating that the difference in their ability to control leishmaniasis is not caused by alterations in parasite sensing by DC. The induction of DC maturation by *Leishmania* was independent of the presence of T and B cells or granulocytes, as it was also observed in $RAG^{-/-}$ and GR1-depleted

mice, but was partially abolished in mice lacking MyD88 [65]. Thus, the recently reported requirement of a functional MyD88 transduction pathway for the development of a protective Th1 immune response to *L. major* [66–68] may involve MyD88-dependent DC maturation. The recognition of *Leishmania* via TLR may be mediated by the promastigote surface glycoconjugate LPG which has been demonstrated to bind to TLR2 [68].

A central aspect of the ability of DC to tailor immune responses to pathogens is their potential to release different cytokines depending on the type of microbial stimulus that is recognized. The level of cytokine expression is enhanced by T-cell feedback signals mediated by CD40 ligation. Various reports document that the choice of cytokine production by DC has a profound influence on the host's ability to control infection with *Leishmania* parasites.

32.5.1
The Role of IL-12 Production by Dendritic Cells

The synthesis of IL-12 at the early stage of infection is crucial for the determination of both innate immunity, as it activates NK cells to produce IFN-γ, and the adaptive host response, via selective induction of Th1 cell differentiation. *Leishmania* parasites have been shown to actively inhibit IL-12 production by macrophages [69] and *in situ* analysis first documented that DC are the source of early IL-12 production following *Leishmania* infection [70]. A subsequent study demonstrated that the secretion of high IL-12 levels by human myeloid DC harboring *L. major* was dependent on the interaction of CD40 expressed by DC with CD40 ligand [71]. Furthermore, DC generated from patients with cutaneous leishmaniasis caused by *L. major*, upon loading with parasites, were able to induce a CD40 ligand-dependent IFN-γ response. In contrast to *L. major*, however, the *Leishmania* species responsible for visceral disease (*L. donovani*), and the species associated with persistent cutaneous lesions (*L. tropica*) did not induce CD40 ligand-mediated production of bioactive heterodimeric IL-12p70 by DC, but primed the cells for expression of only the IL-12p40 subunit [72]. The intrinsic differences in the ability of *Leishmania* species to trigger critical DC functions may contribute to the evolution of different clinical forms of leishmaniasis. Species restriction in the interaction of *Leishmania* and DC has also been observed with DC derived from the bone marrow of mice. DC infection with *L. amazonensis*, a cause of cutaneous leishmaniasis in the New World, resulted in upregulated expression of MHC class II and co-stimulatory molecules including CD40. However, in contrast to what has been found for *L. major*, *L. amazonensis* failed to induce CD40-dependent IL-12 production by DC, but rather enhanced their secretion of IL-4 and priming of a parasite-specific Th2 response [73]. Interestingly, exposure to *L. amazonensis* increased IL-4 production and Th2 priming by DC from susceptible mice but not by those from resistant mice.

Analogous to the concept that the disease outcome, that is healing cutaneous versus nonhealing systemic leishmaniasis, may correlate with the *Leishmania* species-dependent induction of IL-12 production by DC, it is an appealing idea that

differences in the IL-12 levels expressed by DC may also account for the host-dependent resistance or susceptibility of inbred mice to infection with *L. major*. However, there is presently no evidence for such a difference. Langerhans cell-like fetal skin-derived DC from susceptible BALB/c and resistant C57BL/6 mice were demonstrated to be phenotypically and functionally equivalent [74]. *L. major* infection of DC from both strains was accompanied by upregulation of MHC and co-stimulatory molecules and induction of IL-12 release, indicating that genetic susceptibility does probably not reflect intrinsic defects of BALB/c DC to respond to the parasites. In contrast, although the $CD11b^+$ $CD8^-$ subset of lymph node DC was shown to be responsible for the priming of $CD4^+$ T cells in both susceptible BALB/c and resistant B10.D2 mice, DC from susceptible mice had a more pronounced ability to polarize naïve $CD4^+$ T cells into Th2 effector cells than DC from resistant mice [75]. This difference did not correlate with different levels of IL-12 expression by the DC, but $CD11b^+$ DC from resistant B10.D2 mice were found to express significantly more IL-1β mRNA than those from BALB/c mice.

DC subpopulations were shown to secrete different levels of IL-12 in response to infection with *L. major*. The $CD4^-$ $CD8^+$ subset of mouse spleen DC was least permissive to infection but produced the highest amount of IL-12 [29]. In the lymph nodes draining the lesions, the DC subsets of susceptible BALB/c mice overall produced higher levels of IL-12p70 than those of resistant C57BL/6 mice [41]. The cells producing the least IL-12p70 were the plasmacytoid DC, although there was a marked increase at 3 weeks of infection for C57BL/6 mice. Together, the observations described above point to a complex regulation of *Leishmania*-induced IL-12 expression and Th-cell polarization by DC that depends on the parasite species, the type of DC and the stage of infection.

32.5.2
Other Parameters that may Govern the Polarization of T Helper Cells

Additional factors may be involved in the induction of Th1 differentiation by DC. For example, the co-stimulatory molecules CD80 and CD86 are required for the development of an early immune response to *L. major* [76], and expression of CD80 was reported to be downregulated on Langerhans cells from susceptible mice but not on those from resistant mice [77].

With regard to cytokines other than IL-12, members of the IL-1 cytokine system have been identified to be produced at higher levels by DC from resistant mice compared with those from susceptible mice [75, 78]. In one study, using $CD11b^+$ DC isolated from draining lymph nodes of *L. major*-infected mice, it was found that the DC from resistant B10.D2 mice expressed much higher mRNA levels of IL-1β, but not IL-1α, than the DC from susceptible BALB/c mice [75]. In the other study, using Langerhans cell-like fetal skin-derived DC stimulated with *L. major in vitro*, DC from resistant C57BL/6 mice were shown to express significantly higher amounts of IL-1α mRNA than DC from BALB/c mice [78]. Furthermore, it was demonstrated that treatment of susceptible BALB/c mice with recombinant IL-β [75] or IL-1α [78] increased the ability of these mice to control infection and shifted

the Th1/Th2 balance towards a protective immune response. The efficiency of IL-1α administration was found to be strictly dependent on the presence of IL-12, indicating that both cytokines act in conjunction [78]. IL-1α and IL-1β bind to the same receptor, IL-1 receptor type I, and mice deficient for this receptor were shown to develop enhanced Th2-like cytokine responses following infection with *L. major* [79]. DC-derived IL-1 has been demonstrated to be important for IL-12-mediated Th1 differentiation [80, 81] and, thus, appears to be involved in the generation of a protective immune response during leishmaniasis.

32.6 Parasite Evasion of Dendritic Cell Function

A distinct feature of parasites is the production of long-lasting chronic infections. Their successful colonization of the host is dependent on their ability to interfere with the development of an effective immune response. As DC play a key role in connecting innate and adaptive immunity, various strategies to modify DC functions provide means to facilitate parasite survival in the host. *Leishmania* have been shown to impair the maturation, cytokine secretion, migration and antigen presentation function of DC (Table 32.2).

In a model mimicking natural infection with *L. major*, the inoculation of small numbers of promastigotes (100–1000, the amount transmitted by infected sand flies) into a dermal site resulted in a silent phase of 4–5 weeks during which no T-

Tab. 32.2 *Leishmania* parasite evasion of DC functions.

Effect	*Mechanism*	*Parasite species*	*Ref.*
Impairment of DC activation	Uptake of live parasites	*L. mexicana*	83
Impairment of DC activation	Uptake of parasites not opsonized with Ab	*L. amazonensis*	25
Induction of a Th2 response	Inhibition of IL-12 production, induction of IL-4 production by DC	*L. amazonensis*	73
Inhibition of IL-12 production	Limitation of IL-12p35 expression	*L. donovani*, *L. tropica*	72
Impairment of APC functions	Inhibition of IL-12 production, inhibition of MHC class II expression	*L. donovani*	84
Inhibition of DC migration	Secreted parasite products	*L. major*	85
Inhibition of DC migration	*Leishmania* LPG	*L. major*	86
Inhibition of DC migration	Defective CCR7 expression by DC	*L. donovani*	48
Inhibition of CD1-restricted T-cell activation	Reduction of CD1 expression by DC	*L. donovani*	89

Ab, antibodies

cell responses were observed [82]. This may indicate an impairment of DC activation during *in vivo* infection. In contrast to what has been found for *in vitro* infection of DC with *L. major* [27, 28], cultured DC infected with *L. mexicana* did not upregulate MHC class II and CD86 expression and failed to secrete IL-12, whereas their activation in response to other stimuli was not affected [83]. Uptake of *L. amazonensis* induced enhanced expression of MHC class II, CD40, CD80 and CD86 by DC, but infected DC from susceptible mice synthesized IL-4 rather than IL-12 and induced a disease-promoting Th2 response [73]. *Leishmania* species-dependent effects on DC cytokine production were also found with human DC. *In vitro* infection with *L. major*, but not *L. tropica* or *L. donovani*, triggered IL-12 secretion by blood-derived DC from healthy donors [72].

The *in vivo* modulation of DC functions has been analyzed using the model of murine visceral leishmaniasis [84]. Upon infection of mice with *L. donovani*, an impairment in the ability of splenic DC to induce allogeneic mixed lymphocyte reaction and present *L. donovani* antigen to specific T cells was observed at 2 months of infection. This defect in the antigen presentation functions of DC correlated with reduced MHC class II surface expression, lack of IL-12 production and their ability to suppress IFN-γ release by *Leishmania* antigen-primed T cells. At the onset of control over parasite replication in the spleen at 4 months post infection, however, the DC functions were found to be restored.

Impairment of the migratory activities of DC is another effective way to manipulate the host's immune response. The motility of splenic DC was shown to be inhibited by secreted products of *L. major* promastigotes [85], and the emigration of Langerhans cells from skin was significantly reduced by the phosphoglycan moiety of *L. major* LPG [86], suggesting that parasite products interfere with the ability of DC to transport antigen to or within lymphoid organs.

With regard to antigen presentation, an exquisite property of DC is their ability to present non-protein antigens to T cells via CD1 molecules which are constitutively expressed by DC. The CD1 family of non-polymorphic molecules consists of group I CD1 molecules, comprising human CD1a, CD1b and CD1c, and the group II molecule CD1d found on both human and murine DC. The best-characterized microbial antigens for CD1-mediated presentation are mycobacterial lipids and glycolipids [87]. These antigens can be recognized by conventional T cells, a subset of γδ T cells and NK T cells. *Leishmania* species also contain abundant glycolipid molecules and it was recently reported that LPG, as well as related glycoinositol phospholipids, bind to CD1d and stimulate CD1d-dependent IFN-γ production by a subset of hepatic NK T cells [88]. However, infection of human DC with *L. donovani* has been shown to downregulate CD1 expression and CD1-restricted T-cell activation by DC [89].

Finally, sand fly components may also contribute to an impairment of the host immune response. Salivary gland homogenate of *Lutzomyia longipalpis*, the New World vector of *Leishmania*, has been shown to decrease the CD40 ligand-induced expression of MHC class II and co-stimulatory molecules by human DC [90] and may thus interfere with their ability to stimulate an adaptive immune response to parasite antigens.

32.7 Dendritic Cells as Tools for Novel Immune Intervention Strategies Against Leishmaniasis

The knowledge of the crucial role of DC in the tuning of immune responses and the availability of techniques for the generation and culture of DC *ex vivo* has led to their use for specific manipulations of the immune system. Most of these pioneering studies have been performed in tumor models, and some strategies are currently being tested in clinical trials, but DC-based immune intervention approaches were also shown to mediate protection against a wide spectrum of infectious diseases caused by viral, bacterial, parasitic and fungal pathogens [91]. Experimental infection of mice with *Leishmania* is a prototype model to explore the factors driving Th1 differentiation *in vivo* and, thus, the knowledge obtained in this system may be of great value for the development of general concepts to treat disorders that are associated with misdirected Th-cell responses. DC are crucial determinants of the Th-cell effector choice and, thus, may be used as tools to dictate Th1 cell development. The overall aim is the targeting of DC *in vivo* with a prophylactic or therapeutic vaccine containing the optimal *Leishmania* antigen preparation, DC activation molecules and DC-specific ligands or promoters for targeting the desired DC subset. For rational design of such immune intervention strategies against human leishmaniasis, however, the complexity of DC immunobiology brings about the necessity for detailed understanding of the cellular and molecular mechanisms of DC-mediated induction of anti-*Leishmania* immunity. Therefore, *ex vivo* approaches in model systems need to be employed to define the parameters for DC targeting in human tissues.

32.7.1 Dendritic Cell-based Vaccination and Immunotherapy

The first studies documenting that DC can serve as a natural adjuvant to induce protective immunity against leishmaniasis were performed in the model of murine infection with *L. major*. A single treatment with Langerhans cells that had been pulsed with parasite lysate *ex vivo* was shown to induce long-lasting protection of otherwise susceptible BALB/c mice against subsequent challenges with virulent parasites [92]. The solid immunity induced by DC-based vaccination was paralleled by a pronounced shift of the cytokine expression towards a Th1-like pattern with high levels of IFN-γ and very low levels of IL-4 and IL-10. In a murine model of visceral leishmaniasis, it was subsequently shown that DC engineered to overexpress IL-12 and pulsed *ex vivo* with soluble *L. donovani* antigens mediated significantly enhanced protection associated with an increased parasite-specific IFN-γ response [93].

The choice of antigen is of substantial importance for the efficacy of vaccination. The antigen preparation should be molecularly defined and it should be possible to manufacture it in a safe and reproducible manner. DC pulsed with a mixture of the recombinant *Leishmania* antigens LACK, KMP-11, gp63 and PSA, or with the sin-

gle leishmanial peptide LeIF, mediated significant levels of protection against leishmaniasis, demonstrating that the development of a DC-based subunit vaccine against leishmaniasis is feasible [94]. In this study, the protective effect was found to depend on DC-derived IL-12 because antigen-pulsed Langerhans cells from IL-12-deficient mice failed to confer resistance. The importance of antigenic peptide selection for DC-based vaccination was confirmed by the finding that DC pulsed with peptide L1 (154–169aa) of *L. major* gp63 induced protection against experimental cutaneous leishmaniasis that was associated with a Th1 response, whereas DC pulsing with peptide L2 (467–482aa) of gp63 resulted in disease exacerbation and a Th2 profile [95].

DC loaded with *Leishmania* antigen can also be employed for immunotherapy of mice with established infections. This has been documented with IL-12-engineered and *L. donovani* antigen-pulsed DC [93] as well as with IL-12-producing and *L. chagasi* antigen-pulsed DC [96], both enhancing the cure of experimental visceral leishmaniasis. In contrast, the intralesional administration of *L. amazonensis* antigen-pulsed DC plus IL-12 did not promote healing of cutaneous lesions although it induced Th1 cell development [97]. Interestingly, combined treatment with *L. donovani* antigen-pulsed DC and the conventional antileishmanial compound sodium antimony gluconate was shown to result in complete clearance of parasites from the liver and spleen and cure of established murine visceral leishmaniasis [98].

32.7.2
Parameters Determining the Efficacy of Dendritic Cell-based Immune Intervention Strategies

The appropriate instruction of DC is critical for their maturation into APC that direct the development of naive T cells toward a Th1 phenotype. This notion is supported by the finding that a mere expansion of the number of mature DC, which can be achieved by treatment of mice with Flt3 ligand, is not sufficient to mediate complete protection against cutaneous leishmaniasis [99]. DC need to be educated in a specific manner to acquire the ability to drive an effective immune response. The criteria usually applied to evaluate the immunostimulatory potential of DC is their induction of cytokines associated with Th1 or Th2 cells *in vitro*. However, it has recently been shown that the immunological characteristics of DC *in vitro* are not necessarily predictive of their *in vivo* immunizing properties [100]. *L. major* antigen-pulsed DC activated by tumor necrosis factor-α, lipopolysaccharide or CD40 ligation, three prototype DC stimuli that are known to trigger enhanced expression of MHC class II and co-stimulatory molecules by DC and evoke DC cytokine production, failed to induce protection against leishmaniasis in susceptible mice. In contrast, mice vaccinated with a single dose of antigen-loaded DC stimulated by exposure to CpG motifs were completely protected and developed an antigen-specific Th1 response. These findings demonstrated that the type of stimulatory signal is critical for activating the potential of DC to induce a Th1 response *in vivo* that confers complete resistance against an intracellular pathogen. In the same

study, it was also revealed that the role of IL-12 depends on the type of DC used for vaccination against cutaneous leishmaniasis. Whereas earlier reports, using Langerhans cells or Langerhans cell-like DC, emphasized the key role of IL-12 produced by the parasite-antigen presenting DC [74, 94], it was shown that the protection mediated by CpG-activated and *Leishmania* antigen-pulsed DC derived from the bone marrow was independent of their ability to release IL-12 [100]. Taken together, critical parameters determining the efficacy of DC-based vaccination against microbial pathogens include the origin and type of DC, the choice of antigen to be used for DC loading and the state of DC maturation and activation (Figure 32.3).

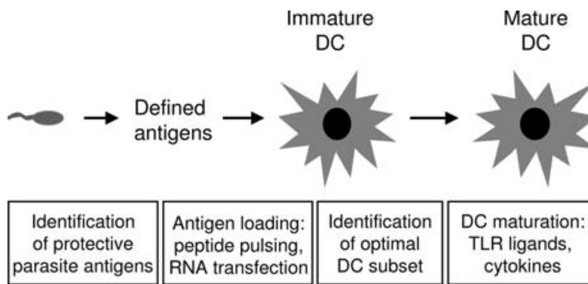

Fig. 32.3 Parameters determining the efficacy of DC-based prophylactic and therapeutic vaccination against leishmaniasis. Single *Leishmania* antigens inducing a protective Th1 response or mixtures of such antigens can be loaded into selected DC subpopulations by peptide pulsing or by transfection with mRNA encoding these antigens. Co-transfection with immunomodulatory cytokines, such as IL-12, may enhance the potential of DC to induce Th1-mediated protection. The type of DC maturation stimulus is another critical factor determining the capacity of DC to instruct protective Th1 cell differentiation.

32.8 Conclusions and Perspectives

Infectious diseases caused by parasites, including leishmaniasis, represent a major world health problem that continues to increase in incidence. Despite considerable efforts, it has not yet been possible to develop any effective vaccine. Moreover, the presently available chemotherapeutic options are unsatisfactory. The rapid progress in the understanding of DC immunobiology and the involvement of DC in the regulation of the *Leishmania*-specific immune response provides a new promise for the development of approaches to manipulate the immune system. DC-based strategies would be of particular relevance to patients in which conventional therapies have failed and to immunocompromised individuals, such as patients with *Leishmania* and HIV co-infections, in which the antimicrobial immune response needs to be enhanced or restored. An ideal scenario would be the antigen loading and activation of DC *in situ*. The approaches currently being explored to target DC in tissues employ DC-specific surface molecules, such as CD205 [101] or

C-type lectins [102], DC-specific promoters [103] and synthetic TLR ligands that specifically interact with DC subpopulations [104]. Additional strategies involve the use of antigen-loaded exosomes derived from DC [105] and antigen delivery by transcutaneous immunization which has been demonstrated to cause the activation of skin DC [106]. Therefore, it is likely that DC-based methods to combat leishmaniasis and other infections caused by intracellular pathogens will become available.

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33
Sentinel and Regulatory Functions of Dendritic Cells in the Immune Response to *Toxoplasma gondii*

Alan Sher, Felix Yarovinsky, Romina Goldszmid, Julio Aliberti and Dragana Jankovic

33.1
Introduction

Toxoplasma gondii is an apicomplexan protozoan that infects felines as its definitive hosts. Cats acquire the parasite through predation of infected rodents and birds. In addition, transmission can occur between these as well as other intermediate hosts in the absence of cats through feeding on infected carrion. Man is an accidental host for *T. gondii* acquiring the infection through contact with cat fecal matter or ingestion of cysts in meat from infected livestock. Most human disease occurs as a consequence of immunodeficiency, typically arising in the unprotected fetus following maternal transplacental infection or in individuals with HIV or drug induced immune suppression [1, 2].

T. gondii promiscuously infects a wide variety of nucleated host cells. Nevertheless, the organism produces an asymptomatic infection in most susceptible immunocompetent hosts due in large part to the rapid induction of strong cell-mediated immune response that results in the control of the rapidly dividing tachyzoite stage and the establishment of chronic infection mediated by dormant bradyzoites. Host resistance to *T. gondii* is critically dependent on IFN-γ produced by NK, CD4 and CD8 T during the acute and chronic phases of infection. TNF-α (TNF) also participates in host resistance, but appears to be more important in the chronic phase where its effector function appears to be closely linked with nitric oxide production [3].

IFN-γ synthesis by NK and T lymphocytes during *T. gondii* infection is critically dependent on IL-12 and mice deficient in the latter cytokine succumb to acute toxoplasmosis with the same kinetics as IFN-γ deficient animals. Since mice develop a strong IL-12 response early in infection, it is logical to presume that the induction of this cytokine is a major initiation signal for host resistance to the parasite [3, 4]. IL-12 is also likely to contribute to Th1 effector choice in *T. gondii* infection although no default to a Th2 cytokine production phenotype is seen in IL-12 deficient animals exposed to the parasite [5].

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While IL-12 is known to be synthesized by a number of different antigen presenting cells as well as neutrophils [6] during *T. gondii* infection, the most relevant source in terms of immune response initiation is likely to be the dendritic cell (DC) and the evidence that the latter cell population produces high levels of the cytokine upon *in vivo* exposure to the parasite provided the first evidence that *T. gondii* is a potent activator of DC function. In this work it was found that injection of live tachyzoites (the rapidly dividing stage of the parasite) or a soluble tachyzoite extract (STAg) into mice results in the rapid appearance within a few hours in spleen of IL-12 p40 producing cells consisting almost entirely of CD11c^{+} DC [7]. Staining of the same spleen sections with anti-CD11c mAb revealed a massive redistribution of DC in spleen stimulated by *T. gondii* injection with most cells leaving the red pulp and marginal zone and clustering in the T-cell areas. In this [7] and in follow up studies [8–10], splenic DC were also shown to be highly responsive to STAg *in vitro* producing significant levels of IL-12 at doses as low as 1 pg ml^{-1} (FY, unpublished observations). Together, the above evidence suggested that the production of IL-12 by DC early in infection is the major "ignition signal" for IFN-γ-dependent host resistance to *T. gondii* infection [11].

These early findings, together with *in vitro* studies characterizing the response of human DC to the parasite [12, 13], laid the groundwork for what has become a highly active research area in which a series of central issues dealing with the role of DC in the immune response to *T. gondii* are being addressed. This chapter presents a brief review of the accumulated data on this topic while focusing on the unique features of the *Toxoplasma*/mouse model as a system for studying DC-pathogen interactions.

33.2
Activation of DC by *T. gondii*

33.2.1
Responsive DC Subpopulations

While as noted above, *T. gondii* can provide a potent stimulus for DC function, it is becoming clear that such parasite-driven responses occur under a limited set of conditions. The best stimulation is observed in the model described above in which murine splenic DC are exposed *in vivo* or *in vitro* to live tachyzoites or STAg. In that system it was shown that the responding IL-12 producing DC belong predominantly to the CD8α^{+} subset [7]. Indeed, bone-marrow (BM)-derived DC which are deficient in this subset respond poorly to *T. gondii* stimulation (JA, FY, unpublished observations). The IL-12 response of splenic CD8α^{+} DC to STAg *in vivo* was found to be extremely rapid, peaking in 3–6 h and was accompanied by a concomitant increase in cell surface CD40 expression [9]. An immediate fall-off in the response was then observed with a reduction to baseline by 24 h. Why CD8α^{+} DC selectively respond to this form of stimulation is unclear. The simplest hypothesis is that they uniquely express the pattern recognition receptors which interact with

STAg ligands. It should be pointed out however that under certain conditions CD8α^- DC can be triggered to produce IL-12 in response to STAg [8] so this restriction is not absolute.

Although in the initial work in the murine system live tachyzoites were found to be equivalent to STAg in their IL-12 inducing potential, there is some controversy as to whether DC infected with the parasite (rather than exposed to released tachzoite products) are able to mount a cytokine response. This is based on the observation (discussed further below) that infection of immature BM DC with tachzoites inhibits their maturation and cytokine production induced by LPS or CD40L [14].

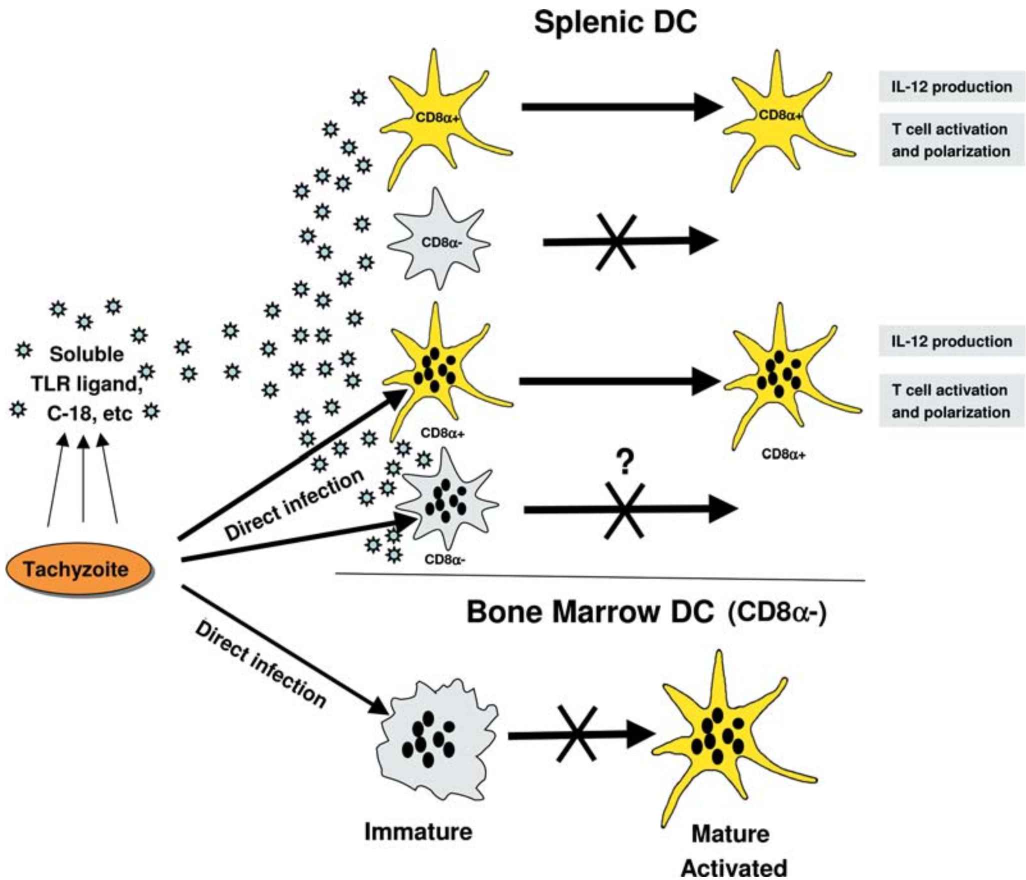

Fig. 33.1 Encounter of DC subsets with *T. gondii* leads to distinct outcomes. CD8α^+ but not CD8α^- DC in spleen and other tissues respond to soluble products released by tachyzoites and present in STAg. Whether intracellular tachyzoites (as opposed to soluble parasite products) can trigger responses in CD8α^- DC, particularly after extended incubation, remains to be determined. While direct infection does not appear to influence the activation status of splenic DC, bone-marrow-derived DC not only fail to respond to soluble tachyzoite Ag but when infected with the parasite display a block in maturation and response to exogenous stimulation with LPS or CD40L [14].

While this inhibition is clear cut, it should be noted that BM DC are poorly responsive in terms of IL-12 production even to STAg so the effect of live infection on parasite-induced synthesis of the cytokine is likely to be minor. In contrast, when splenic DC (which represent a more mature population) are exposed to tachyzoites both parasite-infected and non-infected cells respond with vigorous IL-12 production (RM, unpublished). Taken together these observations suggest that while *T. gondii* infection inhibits DC maturation and responsiveness to exogenous non-parasite activation stimuli, already mature DC can nevertheless be stimulated to respond by soluble parasite products regardless of their infection status (Fig. 33.1). Immune response initiation must therefore depend on the latter DC population.

33.2.2
Host Receptors and Parasite Ligands Involved in Triggering of Murine DC

Most studies investigating the ligand–receptor interactions involved in DC activation by *T. gondii* have utilized the STAg/murine DC model because it provides a soluble extract from which to fractionate parasite molecules that stimulate DC function. As mentioned above, the IL-12 response of splenic DC to STAg is unusually potent exceeding that of LPS, CpG oligonucleotides and other stimuli commonly used to trigger pro-inflammatory cytokine production. This implies either the involvement of a novel signaling pathway or the use of additive or synergistic pathways by multiple ligands in the parasite extract. Another distinctive feature of this system is that the IL-12 inducing activity in STAg is protease sensitive [15] suggesting a critical function for protein ligands.

The role of an unconventional signaling pathway in STAg induced DC activation was initially suggested by studies investigating the role of chemokines in the migration of DC to splenic T-cell areas following STAg injection. It was found that spleens of mice lacking the CC chemokine receptor CCR5 not only display impaired DC migration but also exhibited diminished DC IL-12 production [10]. Moreover, DC from naïve CCR5 KO mice showed reduced IL-12 responses when stimulated with STAg in vitro. In agreement with these observations CCR5 KO mice displayed decreased IL-12 and IFN-γ production following live *T. gondii* infection [10].

Since endogenously produced host CCR5 ligands do not possess significant IL-12 inducing activity, it was logical to suspect the involvement of a parasite ligand in this response. This was confirmed when a *T. gondii* molecule with both IL-12 inducing and CCR5 binding activity was identified in tachyzoite supernatants and STAg. This 18-kDa protein was shown to be an isoform of *T. gondii* cyclophilin (C-18) [15]. Cyclophilins are chaperone-like molecules that possess peptidyl-prolyl isomerase activity and bind the drug Cyclosporin A which inhibit this enzymatic function. C-18, unlike many cyclophilins (i.e. human Cyclophilin A) is a secreted molecule. Its release by tachyzoites or infected cells would explain its ability to trigger DC without directly infecting them [15].

Recombinant C-18 was shown to bind to CCR5 with moderate affinity and trigger chemokine receptor signaling as measured by Ca^{++} flux [15]. No CCR5 binding or IL-12 inducing activity is displayed by closely related cyclophilins including the

homolog from *P. falciparum* [15] and mutated C-18 molecules which no longer bind to CCR5 fail to induce IL-12 [16]. The latter observations suggest that CCR5 binding by C-18 represents an example of molecular mimicry employed by *T. gondii*. Interestingly, in the same-site directed mutagenesis study the peptidyl-prolyl isomerase activity of C-18 was also shown to be necessary for IL-12 induction suggesting that enzymatic modification of CCR5 itself or another host signaling element may be a component of this mechanism [16].

Although recombinant C-18 possessed significant IL-12 inducing activity it was clearly considerably less potent than the starting tachyzoite material from which it was fractionated. Since the *E. coli* expressed protein retains its peptidyl-prolyl isomerase activity, this was unlikely to be due to improper refolding. Instead, the data pointed to the existence of other important IL-12 inducing ligands missed during the initial fractionation of the parasite material that might function together with C-18 in inducing high level DC activation [15].

Because of their major role in microbial recognition it was logical to test the involvement of receptor pathways involving the TLR/IL-1R superfamily in triggering both DC IL-12 production and host resistance to *T. gondii*. To do so mice lacking MyD88, an adaptor molecule used by most TLR as well as IL-1R and IL-18R were employed. DC from these animals showed a near complete abrogation of the STAg induced IL-12 response and when challenged with *T. gondii* the KO animals displayed a loss in resistance to infection equivalent to that of IL-12 deficient mice [17]. Since DC from IL-18R mice show normal STAg induced IL-12 production (T. Kaisho and A. Sher, unpublished observation) and IL-1R antagonists fail to block the DC IL-12 response (JA, unpublished), the observed MyD88 dependency is likely to reflect TLR involvement. TLR-2 has been shown to contribute to the host resistance of mice to very high challenge doses of *T. gondii* [18] as well as to STAg induced chemokine production by neutrophils [19]. Nevertheless, TLR-2 deficient DC as well as neutrophils produce normal amounts of IL-12 following STAg stimulation and TLR-$2^{-/-}$ mice fully resist more physiological low dose infections [17, 19, 20].

A major clue in identifying the TLR involved in DC activation by *T. gondii* came from the initial observation that the relevant molecules are protease sensitive. Indeed, when the search for parasite ligands stimulating DC IL-12 production was continued, this time using stimulation of CCR$5^{-/-}$ DC as the read-out to avoid the detection of C-18, a second much more potent IL-12 inducing protein was identified [21]. This 17.5-kDa molecule (PFTG) was then tested for its ability to stimulate cell lines transfected with either TLR-5 or TLR-11, the two TLR known to recognize proteins. Only the TLR 11 cells responded to PFTG and a similar response was observed with unfractionated STAg. Subsequent, testing of DC from TLR$11^{-/-}$ mice confirmed the requirement for TLR11 in both STAg and PFTG induced IL-12 production *in vitro* as well as *in vivo* [21]. Based on this new data, it would appear that DC activation by *T. gondii* depends on two signals, a major MyD88-dependent signal provided largely by PFTG-TLR-11 interaction and a second weaker enhancing signal resulting from CCR5 ligation of C-18 (Fig. 33.2). How these distinct ligands, receptors and signaling pathways interact is presently unclear. Receptor clusters

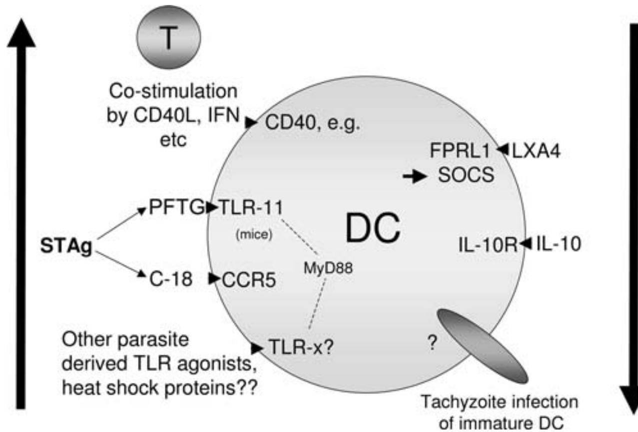

Fig. 33.2 Stimulatory vs downregulatory pathways in the response of DC to *T. gondii*. DC function is stimulated primarily by soluble ligands present in STAg (e.g. PFTG, C-18) although the existence of other agonists in the membrane/insoluble portion of tachyzoites has not been ruled out. These ligands function primarily by triggering MyD88-dependent signaling pathways although an amplifying function for CCR5-dependent signaling stimulated by C-18 has also been demonstrated. TLR-11 has recently been identified as a major TLR in STAg (PFTG)-mediated, MyD88-dependent signaling of murine DC. Other TLR may also participate in mice and those involved in triggering of human DC remain to be identified. DC function following *T. gondii* exposure has been shown to be down regulated by two apparently distinct pathways, the first involving the lipoxin LXA4 and the second IL-10. In addition, parasite infection of immature DC can inhibit the maturation of these cells and their responsiveness to exogenous stimuli.

containing CXCR4 in association with TLR-4 have been observed following LPS stimulation of macrophages [22] and one possibility is that the two *T. gondii* ligands and their corresponding TLR and chemokine receptors stimulate DC by forming a similar membrane complex.

33.2.3
Activation of Human DC

Although much less-well studied, the response of human DC to *T. gondii* appears to have requirements distinct from those described for murine DC. Thus, in two studies involving peripheral blood monocyte derived DC, induction of IL-12 p70 by *T. gondii* was shown to require CD40-CD154 signaling [12, 13], a co-stimulatory mechanism previously shown to enhance, but not to be essential for, murine DC activation [9]. This signal could be provided by co-incubation with $CD4^{+}$ T cells from seropositive donors. Interestingly, in one of these studies [13], in striking contrast to the murine data, direct infection of the DC was found to be required both for the upregulation of co-stimulatory molecule expression and their CD40L-dependent cytokine expression. Taken together both papers suggest that DC acti-

vation in humans requires the presence of pre-existing *T. gondii* specific T cells thereby arguing against the DC as the "ignition signal" for the response of humans against the parasite. An important caveat to this hypothesis is that the DC studied in humans and mice represent distinct subpopulations, the human cells being blood derived and lacking an equivalent of the highly responsive CD8α^+ subset studied in mouse spleen. A further complication is that humans do not appear to have a homolog of the TLR11 molecule recently implicated as a major determinant in the responsiveness of murine DC to STAg [21, 23] and may require other parasite ligands and signaling pathways such as those involving heat shock proteins [24]. Such comparisons may be of interest in understanding the evolution of the host–parasite relationship in *T. gondii* since *Toxoplasma* is a natural parasite of rodents and does not depend on human infection for its transmission [2].

33.3 Regulation of DC Activity

The responsiveness of DC is dramatically downmodulated following infection with tachyzoites or exposure to parasite products. This regulation is likely to serve the parasite by preventing clearance of the organism while protecting its host niche from the potentially lethal immunopathologic effects of an uncontrolled inflammatory response. As already introduced above, infection of murine bone-marrow-derived DC appears to both block their maturation and ability to be activated by LPS as well as present the model Ag OVA to CD4$^+$ T cells [14]. A similar suppression of responsiveness has previously been documented in resting macrophages infected with *T. gondii* and linked with an inhibition in nuclear translocation of NF-κB and STAT-1 [25]. It is likely that parallel mechanisms underlie the induction of non-responsiveness in DC.

Suppression of DC function following *T. gondii* stimulation has also been documented *in vivo* in more mature tissue DC populations. As noted above, the IL-12 response of splenic DC to STAg inoculation is short-lived returning to baseline by 24 h. This reduction in IL-12 production occurs concurrently with the development of nonresponsiveness to secondary administration of STAg, a phenomenon which was referred to as "DC paralysis"[8]. A trivial explanation would be that DC paralysis results from the induction of IL-10, a downregulatory cytokine known to be induced by *T. gondii* and which protects infected mice against IL-12 driven tissue inflammation [26]. Nevertheless not only does DC paralysis not require IL-10, but its induction can actually protect IL-10 deficient mice from lethality following subsequent live *T. gondii* challenge [8].

Based on the evidence that DC paralysis is accompanied by down regulation of CCR5, a receptor which as described above participates in STAg induced IL-12 production, a novel mechanism was identified that may explain the loss in DC responsiveness observed following STAg inoculation. This mechanism involves the induction by STAg or live *T. gondii* infection of lipoxin (LX) A_4, an arachidonic acid

metabolite generated by a 5-lypoxygenase (LO)-dependent pathway. LXA_4, which is thought to signal through the receptor FPRL1 [27] , had previously been demonstrated to downmodulate CCR5 [28]. In the *T. gondii* studies, an LXA4 analog was found to inhibit STAg induced IL-12 production by DC both *in vivo* and *in vitro* an effect which correlated with reduced CCR5 expression [29]. Moreover, STAg was found to be potent stimulus of LXA_4 synthesis *in vivo*. Since this response was found to be 5-LO-dependent, 5-LO deficient mice were then used to examine the role of LXA_4 during *T. gondii* infection. Consistent with the studies employing STAg stimulation *in vivo*, infection of wild-type mice with live parasites induced 5-LO-dependent LXA_4 production [30]. This response was absent in the 5-LO-deficient mice which exhibited enhanced IL-12/IFN-γ synthesis and reduced parasite (brain cyst) counts but succumbed by 28 days post-infection probably as a consequence of an uncontrolled pro-inflammatory response. The role of LXA_4 in these effects was confirmed in reconstitution experiments using an analog of the mediator with a longer clearance time. *T. gondii* infected 5-LO-deficient mice showed enhanced IL-12 production by $CD11c^+$ as well as $CD11c^-$ cells in brain sections suggesting that *in vivo* LXA4 regulates IL-12 production by other APC in addition to DC [30].

In more recent studies the mechanism by which LXA4 inhibits DC IL-12 responses has been investigated and shown to require suppressor of cytokine signaling (SOCS) but utilizing a pathway independent of that involved in SOCS-3-dependent suppression of DC function by IL-10 (F. Machado and J. Aliberti, unpublished).

33.4
Role of DC in *T. gondii*-induced Immune Polarization

Host control of parasite growth during both the acute and chronic phases of *T. gondii* infection depends on IFN-γ secretion by NK and T cells, rather than on cytotoxicity-based effector functions [3] and both $CD4^+$ and CD8T cells from infected mice and humans display a highly polarized Th1 cytokine production profile. As major sentinels of the innate immune system that interact directly with differentiating T cells, it is logical to hypothesize that DC play an important role in establishing this Th1 polarized state early in infection.

The increased survival of *T. gondii*-infected SCID versus IFN-γ deficient animals [31] clearly demonstrates autonomous and potent activation of the innate immune system by this parasite resulting in temporary control of tachyzoite growth. This response, as discussed above, is likely to be directed by IL-12 producing DC that in turn promote IFN-γ synthesis by NK cells. In T-cell sufficient mice, it has been generally assumed that IL-12 serves as a bridge between innate and adaptive immunity by promoting the development of Th1 effector cells [6, 32, 33] thus ensuring lasting control of infection. Consistent with this concept is the finding that mice deficient in the p40 subunit of IL-12 succumb to acute infection with the same kinetics as infected IFN-γ KO mice [34]. To directly address the role of IL-12

signaling in *T. gondii* induced Th1 polarization in a setting which allows host survival, WT and IL-12p40 deficient mice were repeatedly inoculated with radiation-attenuated tachyzoites or STAg. Although Th1-type cytokine production was diminished in the absence of IL-12, the pathogen-specific $CD4^+$ T cells that emerged nevertheless displayed an IFN-γ-dominated lymphokine profile and failed to default to a Th2 phenotype [5]. The same pattern of cytokine expression in Th lymphocytes was observed in STAg-immunized mice deficient in CCR5 or mice doubly deficient in IL-12 and IL-18 (D Jankovic, unpublished data). Additional studies have failed to reveal a requirement for either STAT-4 [35] or IL-27 [36] in *T. gondii* induced Th1 polarization. Together these findings argue that although clearly promoting the expression of IFN-γ during *T. gondii* infection and critical for host resistance, IL-12 is not essential for Th1 effector choice nor are its sister cytokines IL-23 and IL-27. Indeed, a protective function could be revealed for the Th1 cells that emerge in the absence of IL-12 in mice doubly deficient for both IL-10 and IL-12 [5].

The development of *T. gondii*-specific Th1 cells in the absence of IL-12 production suggests that signals distinct from IL-12 are critical for microbial induced Th1 effector choice. That DC can supply these signals was demonstrated in *in vitro* experiments in which highly purified splenic $CD11c^+$ DC exposed to STAg were shown to efficiently direct naïve DO11.10 transgenic $CD4^+$ T cells towards a Th1 phenotype in the presence of nominal OVA peptide [37]. Importantly, this Th1 biasing occurred efficiently with either wild-type or IL-12 deficient DC consistent again with an IL-12-independent mechanism of Th1 differentiation. Nevertheless, DC from $MyD88^{-/-}$ mice failed to induce Th1 polarization in this system suggesting a major role for TLR-dependent recognition and activation [37]. Also, in common with IL-12 production, Th1 polarization by STAg stimulated DC was restricted to the $CD8\alpha^+$ subset with neither bone marrow derived DC, B cells or macrophages functioning in the assay (S. Steinfelder and D. Jankovic, unpublished). A role for $CD8\alpha^+$ DC in *T. gondii* induced Th1 differentiation *in vivo* was suggested by the observation that T-cell cytokine secretion defaults to a Th2 pattern in *T. gondii*-infected ICSBP-deficient mice [38] that display an impairment in $CD8\alpha^+$ DC generation [39]. Thus, activation of DC for Th1 polarization appears to involve the same DC subset and upstream TLR-dependent signaling pathway as that involved in IL-12 production yet does not require the production of that cytokine.

The encounter of microbes or their products with DC can lead to the upregulation of MHC class II as well as co-stimulatory molecules (e.g. CD40) that together should result in APC–T-cell interaction at the high signal strength level previously shown to be associated with Th1 polarization [40]. Such effects of microbial stimulation on DC are likely to occur independently of IL-12 signaling. For example, the redistribution of dendritic cells in spleen induced by *in vivo* STAg injection is unaltered in IL-12-deficient mice and when stimulated with STAg *in vitro* dendritic cells from these animals show normal upregulation of CD40 as well as chemokine production (D Jankovic, unpublished observations). Thus, there are numerous candidate Th1 polarization signals delivered by DC that would require MyD88 sig-

naling (presumably through PFTG-TLR11 interaction) but occur independently of IL-12 production and the identification of those functionally important is a major goal of current research in this area.

33.5 Mechanisms of Antigen Presentation to T Cells

DC are generally assumed to be highly efficient antigen presenting cells in the response to invading pathogens. Nevertheless, in the case of intracellular parasites such as *T. gondii* that reside in vacuoles and modulate host cellular function, the mechanisms by which DC process and present antigen to T lymphocytes are likely to be complex. A further problem has been the failure to identify a dominant TCR in the response to *T. gondii* that can be used for generating TCR transgenic T cells to assay the activating capacity of DC exposed to the parasite. However, the recent development of recombinant *T. gondii* strains expressing OVA [41, 42] has provided an alternative approach to this problem.

The requirements for antigen presentation to $CD4^+$ T cells have been investigated with bone marrow DC infected with OVA transfected parasites or with splenic DC exposed to STAg or tachyzoites as a consequence of short-term *in vivo* inoculation. In the former studies [41], a comparison of recombinant *T. gondii* expressing OVA in either a cytosolic or secreted form detectable in host parasitophorous vacuoles revealed that activation of OVA-specific TCR transgenic $CD4^+$ T cells occurs only when the DC are infected with the antigen secreting parasite strain. The T-cell responses induced were vigorous and comparable to those obtained with OVA peptide pulsed DC with no evidence of parasite induced suppression of DC function as observed in the work discussed above [14].

As an extension of the work on splenic DC activation in the STAg model, Yarovinsky and colleagues have recently examined the ability of DC populations primed *in vivo* by STAg inoculation to activate STAg specific $CD4^+$ T cells in vitro. They observed that splenic DC acquire the capacity to present Ag with the same kinetics as their expression of IL-12 and that in common with both IL-12 production and Th1 polarization, the DC mediating this function are predominantly $CD8\alpha^+$ and their activity is highly dependent on MyD88 signaling. These findings suggest a role for TLR recognition in promoting efficient processing and presentation of *T. gondii* antigens to $CD4^+$ T cells (F. Yarovinsky, unpublished).

The requirements for DC presentation to $CD8^+$ T cells have been studied primarily with OVA transfected parasites. This work has confirmed that despite the localization of the parasite within parasitophorous vacuoles, *T. gondii* secreted proteins are able to enter the Class I presentation pathway by means of a process dependent on the TAP peptide transporter [42].The latter conclusion was based on studies with OVA transfected parasites as well as experiments using a Cre secreting *T. gondii* transfectant where it was found that infection with this parasite strain results in recombination in the nucleus of infected host cells as detected with a GFP loxP reporter. In additional studies utilizing DC exposed *in vitro* to YFP labeled parasites,

it was shown that actively infected (i.e. YFP^+) cells induce much stronger $CD8^+$ T-cell activity than non-infected (YFP^-) DC arguing against a role of crosspresentation by bystander cells [42].The latter interpretation is supported by recent experiments (RM, unpublished) in which activation of OVA transgenic T cells could not be induced by adding MHC class I incompatible DC infected with irradiated OVA transfected parasites to cultures containing uninfected syngeneic DC. Indeed, in direct contrast to DC presentation to $CD4^+$ T cells which can utilize dead organisms, DC presentation of parasite produced OVA to $CD8^+$ T lymphocytes was shown in the latter work to require DC infection by live tachyzoites.

33.6
Towards an Understanding of DC Function *in vivo*

The findings summarized in this chapter document a special relationship that has co-evolved between dendritic cells and *T. gondii* that is likely to be of benefit to both host and parasite. Clearly, because of its invasion of multiple host cells and rapid spread into different tissues *T. gondii* is an intrinsically virulent and pathogenic organism. The ability of DC to rapidly detect the parasite and establish cellular responses that limit infection can thus be seen as both protecting the host and promoting successful parasite transmission through extended host survival and dispersion. Nevertheless, as emphasized repeatedly above, careful regulation of DC function is required to prevent complete elimination of the pathogen as well as excessive cytokine production leading to tissue pathology.

While consistent with both the biology of *T. gondii* as a persistent infection and our current knowledge of DC responses to the parasite *in vitro* and *ex vivo* in the mouse model, the above scheme remains to be confirmed *in vivo* particularly in the context of infection by the natural peroral route. In the latter mode of infection, tachyzoites would first encounter DC in the lamina propria or Peyer's patches following invasion of the intestinal mucosa and present antigen to T cells either in those sites or in draining lymph nodes following migration. Whether initial priming of DC in the gut leads to a different outcome than systemic priming is not known. Th2 responses have been argued to protect against *T. gondii* in mucosal sites (as opposed to systemically) and in this light it is of interest that DC isolated from mesenteric lymph nodes pulsed with tachzoite extract conferred a protective Th2 response against peroral challenge [43] in direct contrast to similar experiments employing splenic DC [44]. Since *T. gondii* invades multiple sites in the intestine in an unsynchronized manner, the study of DC activation and function during natural infection will require the development of new, more sensitive tools for both detecting DC in tissues and measuring their responses. The use of mice with flourochrome tagged DC associated genes is one such approach that could used to tackle this problem.

Peroral infection although perhaps initially priming Th2 responses ultimately leads to systemic IL-12-dependent protection. An interesting question concerns the extent to which DC remain an important source of IL-12 once the initial re-

sponse to the parasite has been initiated. Although requiring stimulation with higher amounts of STAg or IFN-γ priming, neutrophils [45] and macrophages [20, 46] can also produce IL-12 when exposed to *T. gondii* products and it is possible that these cells take over as the major source of the cytokine later in infection. The role of DC as APC during chronic infection also has not yet been carefully addressed.

T. gondii is known to exist in three distinct genetic subtypes which vary in virulence. Essentially all of the work reviewed in this chapter utilized Type I strains such as RH88 for *in vitro* infection studies and as source of tachyzoite extracts while less virulent Type II strains (e.g. ME49) were used for *in vivo* challenge. Although an initial examination of the IL-12 response of splenic DC to Type I and Type II strains failed to reveal a major difference [20], strain distinctions may nevertheless be an important concern in the interpretation of DC functional data. Further studies are needed to examine this issue and to evaluate whether differences in DC reactivity play a role in virulence determination.

Perhaps the ultimate questions concerning the role of DC in the response to *T. gondii* are whether DC are indeed essential for resistance in the murine experimental model and whether a comparable requirement exists in humans. While the former question could be approached in murine studies using newly developed genetic tools for depleting DC *in vivo* [47] our knowledge of the function of DC in the immune response to the parasite in humans is still at a primitive stage. As discussed above, human monocyte derived DC to not appear to be as reactive to *T. gondii* or STAg as murine splenic DC and require co-stimulation in order to display substantial activation responses. Whether this discrepancy reflects a difference in TLR expression between mouse and human (see above) or our inability to study the appropriate DC subset in humans remains to be determined.

Regardless of the inconsistencies with the mouse data, it may be possible to indirectly assess the function of human DC in host resistance through correlative studies in infected subjects. Considerable regional and household clustering of *T. gondii* infection and disease has been observed in countries such as Brazil that cannot be accounted for simply on the basis of parasite exposure [48] (R.T. Gazzinelli, personal communication). As genetic factors are identified which influence these observed differences in the expression of human toxoplasmosis it will be important to include assays of DC function in the group of parameters measured in the population under study. Since disease is relatively rare amongst infected individuals, the discovery of correlations with defective DC response could provide a platform for a more detailed analysis of the influence of DC function on human susceptibility to this widespread pathogen.

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34
Schistosoma

Andrew S. MacDonald and Edward J. Pearce

34.1
Introduction

It has become clear over the past decade that dendritic cells (DC) play a pivotal role in providing the cues that determine the Th1/Th2 effector function bias of CD4 T-cell responses. The early concept of specialized DC subsets, identified in mice on the basis of differential expression of CD4 and CD8α, being restricted in their ability to induce either Th1 or Th2 responses ("DC1" vs. "DC2") has evolved and developed to the point that we now realize that essentially all DC possess the ability to drive both Th1 and Th2 responses. The crucial element that shapes DC plasticity is now recognized to be the nature of the "conditioning" information imparted by the particular pathogen that the DC encounter [1, 2]. The molecular basis of conditioning is most well understood from studies of the maturation of DC exposed to Th1-inducing pathogens, or more usually, defined pathogen-associated molecular patterns (PAMPs) from these organisms. The Th1-biased nature of the data that had been used to construct the current paradigm of DC maturation raised the obvious question of whether DC are activated and function in a similar way in Th2-dominated settings. Several years ago we set out to address this question in the context of an infection, schistosomiasis, in which the immune response is Th2-dominated.

Schistosomes, the causative agents of schistosomiasis, are parasitic helminths (-worms). About 650 million individuals in 76 countries and territories around the world are thought to be are at risk of infection with these complex metazoan pathogens, with at least 190 million people actively infected, according to recent calculations applied to the 1995 world population estimates [3]. In terms of global clinical impact, an estimated 85% of people infected with schistosomes are thought to be located in the African continent [3]. In this area alone, it has been estimated that up to 300 000 people may die each year due to the consequences of severe schistosomiasis.

Four species, *Schistosoma mansoni*, *S. japonicum*, *S. haematobium* and *S. mekongi*, represent the most important agents of human schistosomiasis. The life cycle of these schistosome species is complex. Sexually mature male and female parasites

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live intravascularly in the portal vasculature (*S. mansoni*, *S. japonicum*, *S. mekongi*) or in the blood vessels around the bladder (*S. haematobium*), and eggs produced by female worms pass out of the body via the intestine or the bladder (depending on where the adult parasites live), and hatch in fresh water to release a motile stage that finds and invades the snail intermediate host. As a result of differentiation and asexual reproduction in the snail, new life cycle stages (cercariae) are produced that leave the snail and sit in the water in wait for the definitive human host, which they infect by direct skin-penetration. After a few days in the skin, these larval schistosomes (schistosomula) enter the vasculature and over the course of several weeks migrate intravascularly to their final niche. At this site, the parasites mature and mate. During this period, which can take 5–6 weeks, the host is exposed to antigens from successive developmental stages of the parasite. Infections with schistosomes are chronic and although the immune response eventually becomes capable of preventing superinfection (Reviewed in [4]), it is not able to clear established organisms. Pathology during infection is the result of the host's CD4 T cell-mediated granulomatous response to tissue-trapped parasite eggs.

The relative ease with which *S. mansoni* can be maintained in the laboratory, coupled with the susceptibility of the mouse to infection with this parasite species, has provided an accessible system for studying schistosomiasis. Work with this model has revealed that the dominant immune response to these organisms is Th2-like, and that Th2 cells permit host survival but also contribute to the development of the granulomas and fibrosis that envelope trapped eggs [4, 5]. In early experiments monitoring the time-course of Th2 response development during infection it became apparent that the initial stage of infection during which immature parasites migrate from the skin site of entry to the portal vasculature, is associated with a weak Th1 response, and that the Th2 response develops coincidently with the sexual maturation of the parasites and the onset of egg production [4]. Subsequent work has shown that eggs isolated from infected animals induce marked Th2 responses when injected into naïve animals [4]. Importantly, an extract of eggs, SEA (schistosome egg Ag) mimics whole schistosome eggs in that it is also potent at driving Th2 development after injection into naïve mice, even without the need for co-administration of adjuvant. More than this, SEA, like some other helminth products or extracts [6, 7], could itself be described as an adjuvant, being able to promote Th2 development to model antigens [8, 9]. SEA is known to contain the antigens that are secreted by eggs, and which both induce and are the target of the Th2 response under physiological conditions.

34.2
DC Response to Schistosome Ag

The establishment of a link between the ability of pathogens to stimulate IL-12 production by DC and to induce Th1 responses led to the conclusion that DC not only induce T-cell activation by presenting antigenic peptide/MHC complexes in the context of appropriate co-stimulatory signals, but also provide an additional "third"

signal that ensures the right type of immune T-cell response develops within a given context [10]. Moreover, especially following the publication of micro-array analyses of gene expression in DC exposed to viral, bacterial, or fungal organisms [11], it is clear that these cells are capable of initiating large scale changes in gene expression in response to PAMPs, and that different pathogens can elicit responses that have both specific elements, and general elements common to the response to other types of pathogens. Thus the “third” signal could comprise the coordinated expression (or repression) of multiple relevant genes. In light of these developments, we were interested in examining the effects of SEA, an inherently Th2-inducing antigen, on DC. We anticipated that SEA would induce some degree of activation, perhaps characterized by the expression of IL-4, a gene known to play a role in Th2 cell development. However, our initial analyses of the expression of cytokines and co-stimulatory molecules by murine bone-marrow-derived DC exposed overnight to SEA or dead *S. mansoni* eggs failed to reveal any sign of activation or maturation (Fig. 34.1) [12]. Specifically, we could find no evidence, either at

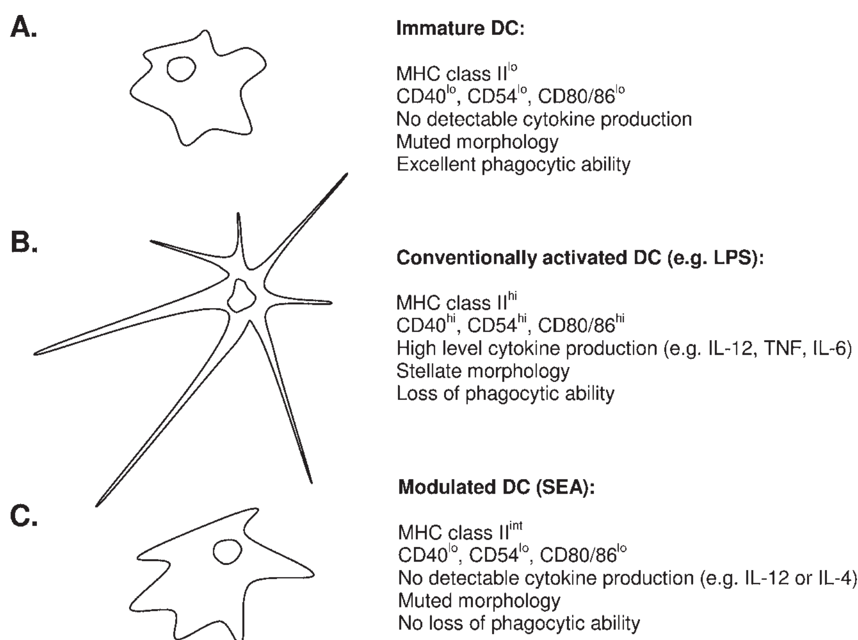

Fig. 34.1 The response of DC to schistosomes. (A) Immature DC are specialized at capturing Ag, but display only low-level expression of molecules such as MHCII or CD80/86, and are thus relatively poor at stimulating naïve T cells. (B). On activation or “maturation” with conventional stimuli such as LPS, DC dramatically transform their morphology to the archetypal stellate shape, lose their Ag capturing ability but concurrently up-regulate expression of surface molecules such as MHCII and CD80/86 and cytokines such as IL-12. As such, these conventionally activated DC are extremely potent at activating naïve T cells. (C) DC exposed to SEA have similarity to immature, rather than conventionally mature, DC. In comparison to immature cells SEA-treated DC display only minor surface MHCII upregulation, retain the ability to phagocytose (unpublished data), and fail to produce detectable cytokine.

the protein or mRNA level, for significant up-regulation of activation markers such as CD80, CD86, CD54 or CD40 or production of cytokines such as IL-12 (p40 or p70), IL-10, IL-6, or TNF. Indeed, the only evidence that we could find for a response of the cells to SEA was a minor up-regulation of surface MHC Class II expression. This contrasted markedly with the effect of *E. coli* LPS or heat killed *Propionibacterium acnes* (a gram$^+$ bacterium) on DC, which we found to be as expected, inducing strongly increased expression of most of the markers examined. Notably, we have never observed IL-4 production by DC, no matter which stimulus we have examined (data not shown). Comprehensive micro-array analyses of gene expression in DC indicate that the expression of very few genes is changed in response to SEA, and analyses of those few that are affected have revealed no currently appreciated links to Th2 response induction [13].

Based on the available data at the time (our study and another which had shown a similar lack of response of murine bone marrow DC to an Ag derived from the parasitic nematode *Acanthocheilonema viteae* [14]) we proposed that a lack of conventional DC activation might be a feature of DC responses to helminths, and perhaps of Th2 development in general. Follow-up studies in other helminth systems have prompted a reassessment of this original idea, indicating that gastrointestinal nematodes such as *Toxocara canis* [15] and *Trichuris muris* (Richard Grencis, personal communication) may "selectively" mature DC, inducing up-regulation of some, but not all, of the hallmarks of conventional activation. However, it needs to be stressed that a complicating issue of these types of studies is that the preparation of Ag from helminths first requires the isolation of the helminth life stage of interest from their hosts or, in some cases, from the environment, and thus any work with these pathogens must include careful controls to rule out the possibility that observed effects on DC are due to contaminating PAMPS such as LPS. In the case of the SEA that we have prepared, due to what we believe are important precautions taken during the preparation of the Ag, which include the inclusion of polymyxin B in the buffers used to isolate the eggs, and the final passage of the isolated eggs through a Percoll density gradient, we have routinely failed to identify any contamination with LPS. Recent work has indicated that SEA not only fails to conventionally activate DC, but additionally is able to induce the downregulation of the steady-state expression levels of some genes [16], and suppress the activation of DC induced by TLR ligands such as *E. coli* LPS, *P. acnes,* CpG, poly I:C or soluble *T. gondii* [13, 17, 18]. This is in part due to the fact that SEA promotes TLR ligand-induced production of IL-10 [13]. However, SEA inhibits TLR-initiated DC activation even in situations where IL-10 is absent. Although initial analyses focused on the ability of SEA to inhibit IL-12 production, or co-stimulatory molecule upregulation, detailed analyses have revealed that SEA inhibits TLR-initiated MAPK and NF-κB signaling, and micro-array based analyses show significant IL-10-independent effects of SEA on the expression of over 100 LPS-regulated genes [13]. These findings indicate that SEA exerts potent anti-inflammatory effects by directly regulating the ability of DC to respond to pro-inflammatory TLR ligands.

How SEA is able to suppress TLR-initiated signaling is the focus of ongoing research. One possibility is that SEA ligates receptors that initiate anti-inflamma-

tory pathways. A candidate for such a receptor is the C-type lectin DC-SIGN or its mouse homolog. DC-SIGN was recently shown to recognize the major Lewis^x[Galβ1,4(Fucα1,3)GlcNAc] glycan epitope of SEA [19]. This is of considerable interest, since binding of the *Mycobacterium tuberculosis* component manLAM to DC-SIGN has been shown to inhibit the production of IL-12, and promote the production of IL-10 [20].

One caveat to the accumulating data indicating muted activation of DC by schistosomes has been presented by recent studies focusing on the effect of live schistosome eggs on murine dendritic cells. In these studies, live *S. mansoni* eggs derived from infected hamsters were found to provoke the transcription of a range of proinflammatory genes, including type I IFN (IFNβ) TNFα and IL-12p40, that one might at first glance associate with Th1, rather than Th2 response induction [21]. Recent work has identified double strand RNA, released from eggs and binding to TLR3 on DC, as the likely initiator of this response [22]. It remains to be seen whether dsRNA is also present within SEA, and/or whether the anti-inflammatory mediator in SEA is actually released from living eggs.

The influence of SEA on human DC appears subtly different to that seen with murine cells. Available data suggest the human monocyte-derived DC (mDC) exposed to SEA *in vitro* display a degree of activation at the level of phenotype, although less than that induced by conventional maturation stimuli such as LPS [23, 24]. Similar to what is seen with murine DC, however, human mDC produce little detectable IL-12p70, TNF or IL-10 in response to SEA [23–25].

More refined dissection of which components of SEA are responsible for affecting DC function has revealed that lipid and carbohydrate components may play important roles. Fractionation of the lipid components of *S. mansoni* eggs or adult worms by TEAE-cellulose column chromatography has identified that lysophosphatidylserine (lyso-PS) isolated from either life cycle stage confers on human mDC the ability to skew allogeneic T cells towards Th2 development *in vitro*. Lyso-PS from either stage also appears to induce IL-10-producing T cells in such conditions. Strikingly, the ability of DC exposed to lyso-PS to modulate DC to induce IL-10 producing "regulatory" T cells requires TLR2, whereas induction of IL-4 producing Th2 cells does not [25]. In contrast to this, somewhat confusingly, TLR4 has been implicated in the ability of lacto-*N*-fucopentaose III (which contains the Lewis^x epitope alluded to above)-pulsed DC to promote Th2 cell development [26, 27]. While these reports and that identifying schistosome egg-derived dsRNA as a ligand for TLR3 [22], implicate TLRs in the induction of Th2 responses, it is important to emphasize that the SEA-specific Th2 response can develop in the absence of MyD88 [66], and that the ability of murine splenic DC to be conditioned by SEA to drive Th2 responses *in vitro* is MyD88-independent [16]. Thus if, as several reports now indicate [23, 28], TLRs are involved in the recognition of SEA by DC, they should be envisaged as activating MyD88-independent rather than MyD88-dependent signaling pathways leading to the conditioning of the DC to promote Th2 cell development. This is consistent with the generally accepted notion that MyD88-dependent signaling leads to the development of DC capable of inducing Th1 rather than Th2 responses [29].

Few studies detail the response of DC to other (i.e. non-egg) schistosome life cycle stages. A recent report indicates that secretions of the infective larvae of *S. mansoni* appear to activate or mature murine DC in a more conventional manner than is true for SEA [30], while still conferring on DC the ability to direct DO11.10 Tg T cells towards a Th2 development. It should be born in mind, however, that the prepatent phase of schistosome infection in mice has not been clearly defined in terms of what type of T-cell response dominates, with a strong Th2 response only really becoming evident after the onset of egg laying (see above) [31, 32].

34.3
Th2 Induction by DC in Response to Schistosome Ag

An emerging idea is that DC that are not fully activated (immature or "semi-mature"), may be tolerogenic rather than immunogenic [33, 34]. It has been proposed that this might provide a mechanism for tolerance induction when DC encounter "innocuous" Ag in the periphery in the form of commensal bacteria, or apoptotic cells. However, the universal applicability of this theory has been called into question by studies of the immunogenic properties of DC exposed to schistosome Ag. As introduced above, DC exposed to SEA effectively retain an immature phenotype and are inhibited from responding maximally to co-pulsed inflammatory TLR-ligands. The obvious question that arises given that SEA pulsed DC could arguably be described as immature or "semi-mature" [34] by standard criteria, is: Are these DC immunogenic or tolerogenic?

If fact SEA-primed DC do not tolerize T cells but instead drive a potent Th2 response, following their transfer into naïve recipient mice [12, 35], and *in vitro* during culture with naïve ova-specific T-cell receptor-transgenic T cells [16]. The Th2-driving ability of SEA-pulsed DC is lost when transferred DC are MHC class II-deficient [12], implying that T-cell responses in this system are induced by the injected DC, and not simply by Ag transfer to recipient cells. These kinds of experiment suggest that DC can play a, perhaps surprisingly, autonomous role in provoking SEA-specific Th2 responses, with no requirement for additional innate cell recognition of any of the components of SEA in this process. This is particularly interesting given that a range of other innate cells including mast cells, basophils and eosinophils, have been shown to produce IL-4 rapidly after exposure to schistosome eggs or egg Ag [36–38]. However, eggs can induce equivalent Th2 responses in the presence or absence of mast cells or eosinophils [36, 39], suggesting that IL-4 derived from these innate sources is contributory, rather than central, for the Th2 process in this setting. Together, these points raise the obvious question of mechanism: how do SEA-exposed DC, activate naïve Th cells and provoke Th2 development?

As alluded to above, our initial studies failed to find any evidence, either at the protein or the message level, for IL-4 production by bone marrow DC in response to SEA [12], even though DC IL-4 has been proposed as a mechanism by which fetal mouse skin DC induce Th2 polarization [40]. Subsequently, we and others have

shown that IL-4-deficient DC induce an equivalent SEA-specific Th2 response when compared to wild-type DC, both *in vivo* [41] and *in vitro* [16], formally ruling out a requirement for IL-4 production during Th2 induction in this context. Similar experiments transferring wild-type or gene-deficient DC into wild-type recipients have revealed that neither IL-12 nor IL-10 production by DC influences their ability to drive an SEA-specific Th2 response ([41] and unpublished data).

Much attention has been afforded to the potential regulatory role of endogenous IL-10 produced during DC activation, and how this might influence their subsequent ability to direct T-cell responses [42, 43]. Although DC-derived IL-10 may affect Th-cell polarization in certain settings, we have found that SEA-pulsed IL-10-deficient murine bone marrow derived DC induce equivalent Th2 response development to WT DC after transfer into recipient mice (Perona-Wright and MacDonald, unpublished observations). Moreover, we have also found that when SEA-pulsed DC (whether WT or IL-10-deficient) are transferred into IL-10-deficent recipient mice, Th2 development is significantly reduced in comparison to WT recipient animals, and accompanied by the emergence of SEA-specific IFNγ production (Perona-Wright and MacDonald, unpublished observations). The implication of this is that IL-10 derived from a source other than the initiating DC acts in the SEA-driven Th2 setting to limit IFNγ production. Thus IL-10, rather than directly promoting Th2 development, appears to be acting to constrain Th1 development to SEA.

An alternative possibility is that SEA-primed DC may in fact play no active role in Th2 development, with Th2 response simply emerging in the absence of DC production of Th1-skewing mediators such as IL-12 – the so-called "default hypothesis" [44, 45]. Although some evidence to support this passive model exists, such data has generally been derived from experimental systems using Ag that are not pathogen-derived, so the relevance of this concept must be re-evaluated in the light of current understanding of the important contribution that the type of Ag encountered makes to DC function. The recent description of the powerful ability of SEA to actively interfere with TLR-mediated signaling in DC (see above) discounts a passive default model of Th2 induction to SEA, but does suggest that suppression of TLR-initiated activation events may favor Th2 development. On this basis, perhaps "default" Th2 development should be qualified as "active" (e.g. SEA) or "passive" (e.g. ova), based on whether or not the Ag in question displays any measurable effect (positive or negative) on TLR-mediated DC activation. Additionally, the possibility of a novel DC mechanism that actively promotes Th2 cell development cannot yet be excluded. It is possible that such a mechanism may only be revealed after "secondary" stimulation of SEA-primed DC via CD40. Indeed, we recently reported the surprising finding that CD40-deficient murine DC lose the ability to stimulate Th2 development to SEA [46], even though SEA does not itself actively induce expression of CD40 on exposed DC [12]. CD40-mediated "licensing" of DC function is not a new concept, but has focused on provision of help for CD8 T-cell responses [47, 48], and Th1 amplification through increased IL-12 production [49, 50]. However, in addition to provoking elevated IL-12 production, CD40 signaling can exert a range of activation effects on DC, including enhancing expression of a

wide range of co-stimulatory molecules such as CD80, CD86, CD70, 41BBL and OX40L [49, 51–53]. This prompts the question of what molecules downstream of DC activation via CD40 might be involved in the Th2 induction process to SEA. Although not upregulated on the surface of murine DC after exposure to SEA, OX40L has been implicated in the ability of human mDC to direct Th2 polarization of naïve T cells *in vitro* [24], making this a prime candidate for a molecule that could provide a positive signal from DC for Th2 induction. Whether this is also the case *in vivo* remains to be determined, as does identifying whether OX40L represents the sole positive signal for SEA driven Th2 induction, or one of a team of collaborators. Of considerable interest in this regard is the recent report that the expression of the Notch ligand Jagged by DC is important for their ability to induce Th2 cell development [54], and current work aims to examine Jagged expression on DC exposed to SEA plus or minus CD40 agonist.

As mentioned previously, there are only limited reports of how other (non SEA) Ag influence the ability of DC to direct T-cell responses, and this area requires further work. It appears that while the secretions of mechanically transformed cercariae confer on murine DC the ability to polarize DO11.10 T cells *in vitro* and *in vivo* [30], soluble schistosomulum Ag does not act to do the same when pulsed DC are transferred into naïve recipient mice [35].

It is clear that several factors, including human DC versus murine DC differences, source/subset of DC used, method of DC generation, etc., collude to confound

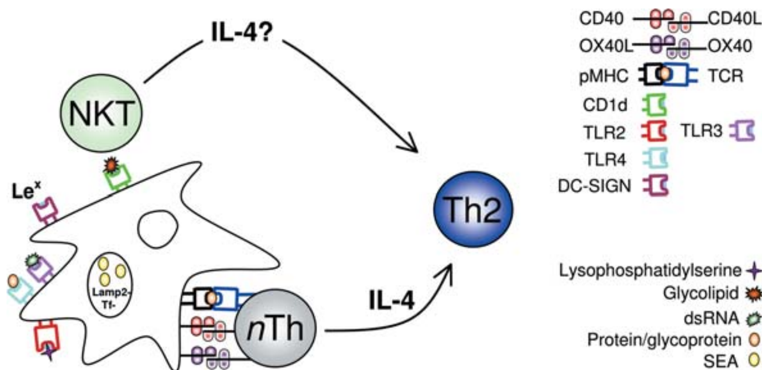

Fig. 34.2 Th2 induction by DC in response to SEA. It is likely that multiple components of SEA bind to a range of pattern recognition receptors on DC, including C-type lectins [19] and TLRs [22, 25, 27]. Although several TLRs have been implicated in this process, MyD88-deficient mice are still capable of making Th2 responses to SEA [66], suggesting either a degree of redundancy in the system or, more likely, that MyD88-independent TLR-initiated signaling is important. Once internalized SEA is distributed to Lamp2^{-}, Transferrin^{-} vesicular compartments within the DC [18]. CD40 expression is critical for DC induction of Th2 response to SEA [46], suggesting downstream involvement of CD40-mediated activation events in this process, with OX-40L being one potential candidate that could fulfill this function. IL-4-deficient DC show no deficiency in Th2 induction to SEA [41], although IL-4 from a source other than the initiating DC is paramount to sustain the developing CD4 T-cell response. NKT cells may provide one source of such IL-4, as CD1d-deficient DC display impaired Th2-induction abilities to SEA [35].

attempts to build a coherent understanding of how DC function during Th2 development to SEA. Perhaps the single most important factor to consider may be whether studies have been carried out *in vitro* or *in vivo*. While *in vitro* DC:T cell coculture experiments undeniably provide a finely controlled system, they exclude the potentially central input and influence of other cell types during the T-cell activation and polarization process [55]. For example, once activated, CD4 T cells migrate to B cell follicles [56], wherein the B cells can provide "help" to support T-cell expansion, effector function and memory formation, via mechanisms such as costimulation [57]. It could be argued that the interaction of T cells with other cell types may not be important during T-cell priming, where DC:T cell dialog has been thought to be key. However, it appears that even in DC-driven Ag-delivery systems (i.e. in the absence of free Ag) innate cells such as NK cells can be intricately involved in T-cell polarization *in vivo* [58]. In relation to this, several recent reports have promoted a role for CD1d-restricted NKT cells in SEA-driven Th2 response development *in vivo* [17, 35]. It is likely, therefore, that during both induction and amplification of the developing T-cell response, a "network" of additional cell types contributes towards the emergent T-cell polarization phenotype.

A summary of the issues discussed in this section is presented in Fig. 34.2.

34.4
DC During Schistosome Infection

Understanding of how DC function during active schistosome infection is still in its infancy. Dissecting the role of DC in the complex setting of an ongoing infection with an organism that has several developmental stages and locations within the mammalian host presents a somewhat daunting challenge. Added to these factors, chronic schistosome infection is in no way as controlled a setting as direct, one-shot Ag administration, with ongoing production and release of eggs providing a constant and accumulating source of Ag. How can we begin to evaluate DC activation and function in this context? The limited data on this topic that does exist has been generated in rodent models of schistosome infection.

A key role has been proposed for cercarial-derived prostaglandins (PGD_2) in preventing migration of Langerhans cells (LC) from the site of larval penetration to the skin-draining lymph nodes during infection of mice with *S. mansoni* [59]. Whether other stages of the parasite exert similar modulatory effects on LC (or other DC subsets) remains to be determined. These results expand upon earlier experiments assessing cellular recruitment in skin-draining lymph nodes of guinea pigs exposed to *S. mansoni* cercariae. In this case, enhanced LC recruitment was seen following percutaneous exposure of guinea pigs to UV-attenuated compared to normal *S. mansoni* cercariae [60], raising the interesting possibility that attenuated cercariae may themselves exhibit impaired PGE_2 production or secretion as well as being less effective at provoking PGE_2 from host skin cells [61].

As yet, studies assessing DC function during infection beyond the early stages of cercarial penetration are limited. We have assessed the activation phenotype of DC isolated from the spleen or MLN over the course of murine infection with *S. man-*

soni, and observed only minor up-regulation of expression of conventional activation markers by both CD8α⁺ and CD8α⁻ subsets, but most marked in the CD8α⁺ cells, that are not apparent in SEA-pulsed bone marrow DC [62]. Another intriguing difference between SEA-pulsed bone marrow DC and DC isolated from the spleens of infected mice is that, while SEA-pulsed bone marrow DC do not make IL-12 following CD40 ligation [12], total CD11c⁺ splenic DC isolated from infected mice are primed to make this cytokine and indeed produce more of it in response to CD40 ligation than do CD11c⁺ splenic DC from uninfected mice [62]. These bone marrow/splenic DC differences could be explained by the heterogenous nature of CD11c⁺ DC isolated *ex vivo*. Splenic CD11c⁺ DC comprise several subsets, not all of which are similar to the CD8α⁻ bone marrow DC most extensively studied in schistosome research [63]. Compared to CD8α⁻ DC, CD8α⁺ DC are more potent IL-12 producers and while both subsets from the spleens of infected mice can be activated to produce IL-12 in response to CD40 ligation, it is likely that it is the CD8α⁺ subset that is capable of making the most IL-12.

The low-level activation phenotype of DC isolated from schistosome infected mice appears to depend upon CD40:CD154 interaction, as it is not apparent in DC from infected CD154$^{-/-}$ mice [62]. Further, maintenance of low-level DC activation status during murine schistosome infections may be controlled, at least in terms of MHC Class II and co-stimulatory molecule expression, by IL-10, since DC isolated from schistosome infected IL-10$^{-/-}$ mice display a hyperactivated phenotype [64]. Together, these data support the idea of a coordinated sequence of events centering on (low level) DC activation mediated by CD40 ligation, and under the ultimate control of IL-10.

Recent developments in the area of intravital imaging, using techniques such as 2-photon microscopy [65], should prove invaluable in furthering our currently rudimentary understanding of DC activation and function during active infection. An important step towards realizing such experiments will be the generation of transgenic mice to be able to track schistosome-specific lymphocytes during infection. Additionally, characterization of human DC isolated from schistosome-infected individuals will be the vital "next step" in extending the conclusions gained from murine studies to a broader stage, and ultimately towards the rational design and development of vaccines and therapies.

34.5 Discussion

How does all of the above information fit together in terms of what is actually likely to be occurring *in vivo* during active schistosome infection? It is becoming increasingly clear that in order to be able to assemble this increasingly complex puzzle, we will first need to gather more pieces. Determining how DC respond, both *in vitro* and *in vivo*, to the many and varied immune stimuli provided by the different schistosome life cycle stages, is the vital first step towards dissecting this process. Although technically challenging, translating that information into understanding its actual influence over effector T-cell fate *in vivo*, a process that is cur-

rently very much the realm of potentially misleading speculation, is the next crucial step that will require thinking outside the 96-well plate. Only once we have a firm grasp of these basic events will we be able to attempt to integrate available information into a unifying model of DC function during schistosomiasis.

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XII
Bacteria

35
Dendritic Cells and Immunity to Salmonella

Mary Jo Wick

35.1
Introduction

An early and necessary event required for pathogen elimination is phagocytosis and destruction of the bacteria. This must be coupled to processing and presentation of bacterial antigens to ensure generation of adaptive immunity. Initiating an adaptive response in a primary infection requires that the bacteria-derived peptides are presented under conditions that lead to activation of naïve T cells, eliciting the appropriate effector function in the responding cells, and developing a memory T-cell pool. This requires sufficient co-stimulation, production of appropriate response-skewing cytokines such as IL-12, and localization in the appropriate place, that is, in secondary lymphoid organs. To achieve this, the cells that initially phagocytize the bacteria must migrate from the peripheral infected tissues to a secondary lymphoid organ.

There is only one cell type capable of performing all of these functions, dendritic cells (DC). DC fulfill a unique niche in immunity to bacterial pathogens and are the cornerstone in the transition from innate to adaptive immunity. This chapter summarizes work underlying the current understanding of the role of DC in the immune response to *Salmonella*. Studies performed *in vitro* that provide insight into bacterial uptake, processing and presentation of *Salmonella* by DC, and DC maturation in response to *Salmonella* are summarized first. Findings on the role of DC during *Salmonella* infection assessed in murine infection models will subsequently be discussed.

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35.2
Dendritic Cell Subsets, Short and Sweet

Although presented in great detail and scope elsewhere in this book, a very brief description of murine DC subsets may be useful here to facilitate discussions of *Salmonella*-DC interactions in this chapter. Conventional DC in mice express high levels of the p150/90 integrin CD11c as well as high basal levels of MHC-II. They can thus conveniently be identified as $CD11c^{hi}MHC\text{-}II^{+}$ cells. These DC from secondary lymphoid organs can be further divided into subsets based on expression of CD11b, CD8α, and CD4, among other molecules [1–3]. For example, conventional DC in the spleen can be divided into $CD8\alpha^{+}CD4^{-}CD11b^{-}$, $CD8\alpha^{-}CD4^{+}CD11b^{+}$ and $CD8\alpha^{-}CD4^{-}CD11b^{+}$ subsets that comprise approximately 25%, 50% and 25% of total $CD11c^{hi}MHC\text{-}II^{+}$ cells in this organ, respectively. In contrast to the spleen, $CD4^{+}$ DC are rare in mesenteric lymph nodes (MLN), Peyer's patches (PP) and the liver of mice. $CD11c^{hi}MHC\text{-}II^{+}$ cells present in these organs can be described as $CD8\alpha^{+}CD4^{-}CD11b^{-}$, $CD8\alpha^{-}CD4^{-}CD11b^{+}$ and $CD8\alpha^{-}CD4^{-}CD11b^{-}$ populations [4–7]. These subsets are present in somewhat different relative proportions depending on the organ [8].

35.3
Dendritic Cells and Salmonella: Lessons from in vitro Studies

35.3.1
Bacterial Uptake and the Fate of Internalized Bacteria

Salmonella enterica Serovar Typhimurium (*S. typhimurium*) is a facultative, Gram negative intracellular pathogen that has developed strategies allowing it to survive in phagocytic cells, both in macrophages and DC, despite landing in what can be a rather harsh vacuolar environment after phagocytosis. Studies using murine DC, either derived from bone marrow cultured in the presence of GM-CSF or $CD11c^{hi}$ DC enriched from the spleen, liver or MLN of normal mice, have demonstrated that these cells can indeed internalize *Salmonella* [7, 9–15]. Internalization requires only a fairly short pulse of DC with bacteria and occurs even at low bacteria to DC infection ratios [7, 9, 10, 12–15]. In addition, active opsonization is not required, although opsonization increases the number of bacteria internalized [11]. With respect to a differential capacity of DC subsets to internalize *Salmonella*, no major differences have thus far been reported. For example, a similar percent of freshly isolated $CD8\alpha^{+}$, $CD8\alpha^{-}CD4^{-}$ and $CD8\alpha^{-}CD4^{+}$ splenic DC were positive for green fluorescent protein (GFP) after co-culture with GFP-expressing *Salmonella* [10]. This suggests no major differences among the splenic DC subsets to internalize bacteria at a given infection ratio. Similar analysis of DC isolated from the liver, however, revealed that $CD8\alpha^{-}$ DC were perhaps slightly more effective than $CD8\alpha^{+}$ DC in internalizing *Salmonella*, while cells separated on the bases of MHC-II expression rather than CD8α showed that $MHC\text{-}II^{hi}$ cells were more efficient at inter-

nalizing the bacteria than MHC-II^{low} cells [7]. Functional consequences of this are presently not known.

Salmonella enter DC in a process that involves actin cytoskeletal rearrangements [7, 10, 16] and reside in vacuolar compartments termed *Salmonella*-containing vacuoles (SCVs) [17]. Unlike other intracellular bacteria such as *Listeria* and *Shigella, Salmonella* remain confined in vacuoles and do not produce a pore-forming protein allowing their active escape into the cytosol. Instead they encode proteins, particularly SifA, that are required to maintain the integrity of the SCVs and keep *Salmonella* physically separated from the cytosol [15, 17, 18]. Thus, only *Salmonella* lacking SifA readily access the cytosol of DC whereas wild type bacteria remain confined in vacuoles [15].

The fate of *Salmonella* after phagocytosis by macrophages has been studied for decades and has resulted in a wealth of knowledge on this topic. For example, *Salmonella* residence in macrophage SCVs diverts the normal maturation of phagosomes, prevents vesicle fusion with lysosomes, and interferes with delivery of antimicrobial effector proteins, such as NADPH oxidase and inducible nitric oxide synthase (iNOS) that otherwise contribute to the demise of the bacteria, to bacteria-containing compartments [17, 18].

In contrast to the extensive literature on *Salmonella* trafficking in macrophages, however, relatively little is known about the fate of this bacterium after phagocytosis by DC. The few studies on the intracellular fate of *Salmonella* in DC available thus far suggest similar manipulation of phagosomes in DC as reported for macrophages. For instance, SCVs in DC are capable of inducing the *Salmonella* genes necessary for altered SCV trafficking and function [14]. In addition, the vacuoles containing *Salmonella* in DC have features consistent with SCVs defined in macrophages, such as the presence of LAMP-1 and MHC-II [14]. However, in contrast to the increase in bacterial number that occurs after *Salmonella* phagocytosis by macrophages, studies from several groups have shown that *Salmonella* do not increase in number after internalization by DC [12, 14, 15, 19]. Although details such as the initial multiplicity of infection used, time frame analyzed and number of bacteria recovered differs in these studies, the consensus appears to be that the number of *Salmonella* in infected DC remains relatively constant over the first 48 h of infection. A recent report further showed that the maintenance of constant bacterial numbers during the first 24 h in SCVs of infected DC is due to the static, non-dividing nature of intracellular *Salmonella* rather than the alternate explanation that bacterial multiplication occurs at a rate equivalent to bacterial killing [14]. Although static in nature, recent gene expression profiling studies suggest that the bacteria in SCVs are not in the stationary growth phase classically defined by *in vitro* culturing of bacteria in liquid medium [20]. These profiling studies were performed on infected macrophages, however, so whether the same is true in infected DC remains to be directly addressed.

The mechanisms underlying the capacity of *Salmonella* to remain in a viable, static state in SCVs of infected DC are not known. What is known from studies in macrophages, however, is that production of reactive oxygen and nitrogen intermediates by inducible nitric oxide synthase (iNOS) and NADPH oxidase, respectively,

have a role in controlling *Salmonella* replication [21, 22]. Indeed, *Salmonella* has tackled this problem by preventing the co-localization of these enzymes to SCVs [22–24]. Precisely how products of these pathways work to control *Salmonella* replication in infected macrophages is complex [25–27]. There is a sequential contribution of an NADPH oxidase–dependent oxidative bactericidal phase followed by an iNOS–dependent bacteriostatic phase. Moreover, the mediators generated by these pathways can interact in several ways and multiple antimicrobial effects are possible [25–27].

The contribution of reactive oxygen and nitrogen intermediates to controlling *Salmonella* growth in DC is not well understood. Murine DC appear capable of producing nitric oxide in response to *Salmonella*, despite some inconsistency in reports where two groups found production [11, 28] and one did not [19]. The precise reason for this discrepancy is not clear. Possibilities include that the infection conditions resulted in different quantities of bacteria internalized per DC and a threshold number of bacteria is required to trigger iNOS, or that the use of opsonized versus non-opsonized bacteria results in altered intracellular targeting of the bacteria which could be linked to a difference in the capacity to divert iNOS localization [24]. Alternatively, different times of bacteria-DC co-culture used or the presence of residual extracellular bacteria in cultures that provided a stimulus that influenced iNOS production may contribute to the observed difference. Nevertheless, murine DC are capable of producing nitric oxide in response to *Salmonella*. Furthermore, nitric oxide produced by DC co-cultured with opsonized bacteria reduced intracellular bacterial yields and had a bactericidal effect [11]. This was in contrast to the effect of nitric oxide produced by *Salmonella*-infected macrophages, which has a bacteriostatic effect [11, 22, 26]. This difference may indicate different environmental conditions in SCVs of macrophages versus DC [25–27].

In addition to their capacity to produce reactive nitrogen intermediates, both murine and human DC have NADPH oxidase components and can generate reactive oxygen products in response to bacterial ligands such as LPS and flagella [29, 30]. Although not yet addressed for *Salmonella*, Vulcano et al showed NADPH-dependent killing of *E. coli* after co-culture with LPS-treated DC [30]. Interestingly, DC appear to have a NADPH oxidase inhibitory factor located in the membrane whose inhibition of oxidase activation is relieved after exposure to microbial components such as LPS [29]. This suggests that DC may exert additional regulatory control on NADPH oxidase activity. Despite these data, however, the role of NADPH oxidase-dependent mechanisms in controlling replication in *Salmonella*-infected DC is not yet established. Likewise, if SCVs of DC have a capacity similar to that seen in macrophages to modulate the localization of NADPH oxidase or iNOS awaits investigation. More detailed studies of the environment of *Salmonella*-containing vacuoles in infected DC and the contribution of NADPH oxidase- and iNOS-dependent control of *Salmonella* replication by DC warrant further study. Moreover, the mechanisms that maintain the viable but nondividing nature of *Salmonella* in DC SCVs [14], and the host and bacterial factors that regulate this, promise to be interesting areas of investigation.

35.3.2 Presentation of Salmonella Antigens by Dendritic Cells

35.3.2.1 Processing of Salmonella for Direct Presentation on MHC-II by Infected Dendritic Cells

CD4 T-cell responses, particularly induction of IFNγ-producing cells, are critical to controlling and eradicating *Salmonella* in infected hosts [31–33]. To initiate $CD4^+$ T-cell responses, antigens must be processed and presented on major histocompatibility complex (MHC) class II molecules (MHC-II). The type of antigens typically processed for MHC-II presentation are exogenous in nature, that is, are extracellular antigens that are taken into a cell by pinocytosis, endocytosis, or phagocytosis, depending on the nature of the antigen. Despite that *Salmonella* reside intracellularly in infected hosts [34–38], they spend part of their time extracellularly, such as before the bacteria are phagocytosed by, or invade, host cells. They also have access to the extracellular environment after death and lysis of an infected cell [39, 40].

It thus follows that antigens from *Salmonella* are processed and presented on MHC-II for recognition by $CD4^+$ T cells. Indeed, immature DC phagocytose and present *Salmonella*-derived antigens on MHC-II to $CD4^+$ T cells, as has been shown using bone-marrow-derived DC [12, 16, 19]. It has also been demonstrated in assays using DC freshly isolated from the spleen, MLN and liver of naïve mice [7, 10]. Presentation of *Salmonella* antigens on MHC-II by *Salmonella*-infected DC requires actin-driven cytoskeletal rearrangements to internalize the bacteria and bacterial passage through acidic compartments [10, 16]. Furthermore, $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC appear to have a similar capacity to internalize and process *Salmonella* for peptide presentation on MHC-II [10]. Thus, DC in the spleen, liver and MLN can internalize and process *Salmonella* for peptide presentation on MHC-II for recognition by $CD4^+$ T cells.

35.3.2.2 Processing of Salmonella for Direct Presentation on MHC-I by Infected Dendritic Cells

As discussed above, wild type (SifA-expressing) *Salmonella* internalized by DC remain confined in vacuolar compartments [15, 17, 18]. However, DC can process internalized *Salmonella* and present peptides derived from bacteria-encoded proteins on MHC-I [7, 10, 12, 16, 19, 41, 42]. At first glance this appears incongruous with respect to the classical MHC-I antigen presentation pathway where endogenous, cytosolic proteins are processed in a proteasome-dependent fashion, transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) and loaded on newly synthesized MHC-I for ultimate display on the cell surface [43]. However, data reported three decades ago showing that exogenous antigens can be presented on MHC-I [44] were the basis for solving this paradox and the starting point for what is now extensive literature documenting and characterizing presentation of exogenous antigens on MHC-I [45–47].

Presentation of antigens by DC that have phagocytosed *Salmonella* has been used by several investigators both *in vitro* and *in vivo* as a model to understand presentation of vacuole-confined bacteria. The relevance of studying MHC-I presentation of *Salmonella* antigens is underscored by the development of $CD8^+$ T cells, in addition to $CD4^+$ T cells, to bacteria-encoded antigens in infected mice and humans [36, 48–53]. Indeed, recombinant attenuated *Salmonella* is a potential vaccine delivery system to elicit CD4 and CD8 T-cell responses to cloned and expressed antigens.

Similar to the results for MHC-II presentation, DC isolated from the spleen, MLN and liver of naïve mice as well as bone-marrow-derived DC are capable of presenting *Salmonella* antigens on MHC-I [7, 10, 12, 16, 19, 41, 42]. Likewise, both $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC subsets internalize *Salmonella* and process the bacteria for peptide presentation on MHC-I [10]. Moreover, despite the vacuolar localization of *Salmonella* after phagocytosis by DC, the data available thus far suggest that components of the cytosolic MHC-I antigen presentation pathway are used. For example, DC have a strict requirement for the TAP peptide transporter to present *Salmonella* antigens on MHC-I [54]. In addition, although not formally demonstrated for *Salmonella*, studies using *E. coli* demonstrate that newly synthesized MHC-I molecules and the proteasome are also required for DC presentation of bacteria-encoded antigens [41]. It thus appears that the cytosolic antigen presentation machinery is used to for MHC-I presentation of bacterial antigens after phagocytic uptake of *Salmonella* by DC. This mechanism appears distinct from that used by macrophages to present *Salmonella* antigens. In the case of macrophages, the TAP transporter and proteasomes are not required [46, 55, 56]. It thus appears that macrophages and DC have different requirements to present *Salmonella* antigens on MHC-I. However, relatively little is known about the pathway(s) used for MHC-I presentation of *Salmonella* antigens by infected DC, and additional experiments that characterize this would be informative. This is particularly true in light of recent findings describing the intersection of the endoplasmic reticulum with phagosomes that results in phagosomes capable of TAP- and proteasome-dependent presentation of exogenous antigens on MHC-I [57, 58].

35.3.2.3 Modulating of Antigen Presentation by *Salmonella*

Salmonella is a facultative intracellular bacterium that has evolved strategies to survive in phagosomal environments, which are otherwise designed to kill phagocytosed microbes. It thus follows that the intracellular survival strategies used by *Salmonella* may influence the capacity of an infected DC to process and present bacterial antigens. Indeed, the *phoP/phoQ* regulatory system, which controls the expression of over 40 genes and is involved in bacterial survival in phagosomal compartments [59, 60], can influence the ability of infected DC to present *Salmonella*-encoded antigens. Antigens from *Salmonella* constitutively expressing *phoP,* so that the set of *phoP*-activated genes are switched on and the *phoP*-repressed genes are off, are more efficiently presented on MHC-II after bacterial phagocytosis by DC [19]. The effect of *phoP/phoQ* was observed when antigen presentation was quanti-

tated after a short (2-h) but not a longer (24-h) exposure to bacteria [12, 19]. The effect of the *phoP* locus on antigen presentation by infected DC was abrogated when the bacteria were heat-killed, demonstrating that bacterial gene expression was required for the effect [19]. Together these data suggest that the *phoP/phoQ* regulatory locus can influence the capacity of DC to present *Salmonella* antigens on MHC-II during a short time frame after bacterial infection. Despite the effect of *phoP/phoQ* on presentation of *Salmonella* antigens on MHC-II by infected DC, an effect of this locus on presentation of *Salmonella* antigens on MHC-I was not detected [19].

Other factors, such as the ease of bacterial uptake and the amount of antigen present in the bacteria that are internalized, can influence the efficiency of antigen presentation. Relatively little data is available that directly addresses these issues for the presentation of *Salmonella* antigens by DC. However, a recent report showed that directing *Salmonella* to Fcγ receptors on DC by opsonization with *Salmonella*-specific IgG enhanced the presentation of a *Salmonella*-encoded antigen on MHC-I and MHC-II [61]. In contrast to previous reports [7, 10, 12, 16, 19, 42], Tobar et al. could only detect presentation of antigens encoded in wild type *Salmonella* when the bacteria were opsonized. Based on this they concluded that virulent *Salmonella* interferes with the capacity of DC to process the bacteria for antigen presentation, and this could be overcome by targeting the bacteria to Fcγ receptors. However, antigen abundance in the two strains compared in the study was not assessed, and direct evidence for active inhibition of antigen presentation by *Salmonella* was lacking [61]. Thus, the alternate explanation that increasing antigen load in the DC by opsonization, perhaps combined with altered intracellular trafficking when internalized as immune complexes, was responsible for the observed presentation cannot be excluded. Indeed, opsonization increases the number of bacteria per DC [11, 62], and targeting antigens to Fcγ receptors on DC has been shown to increase antigen presentation in several other settings [63–66].

35.3.2.4 **Waste not, Want not: Dendritic Cells as Bystander Antigen-presenting Cells**

The presentation of *Salmonella* antigens by DC discussed above focused on direct antigen presentation by infected cells. In this pathway, DC internalize *Salmonella*, process the bacteria and display MHC molecules containing bacterial antigens on their cell surface for recognition by T cells. In other words, the *Salmonella*-infected DC directly process the bacteria and present bacterial antigens to T cells. However, DC can also present bacterial antigens when they themselves are not infected by the bacteria in a process called indirect presentation or, in the case of MHC-I, crosspresentation. In indirect presentation, the DC that present *Salmonella* antigens are not infected by the bacteria *per se*. Instead, the DC are non-infected bystander cells that acquired *Salmonella* antigens by internalizing neighboring cells that have undergone death due to *Salmonella* infection. *Salmonella* expressing the type III secretion system is cytotoxic to infected cells [39], and dead cells can not productively interact with T cells [42]. However, the indirect antigen presentation pathway provides a safety valve where DC “mop up” cell debris containing *Salmo-*

nella antigens and use this material to stimulate T cells [42]. In the case of *Salmonella* and other intracellular bacteria, this property could be very useful to the immune system and allow detection of microbes that could otherwise be elusive [67]. Indeed, bystander presentation of antigenic material from cells induced to undergo apoptosis due to infection with *Mycobacterium tuberculosis* has also been shown [68]. In the case of this vacuole-confined bacterium, apoptotic macrophages shuttle vesicles containing mycobacterial lipids and proteins to DC which in turn present the material to CD1b- and MHC-I-restricted T cells, respectively [68].

Salmonella-induced cell death has been best studied in infected macrophages and epithelial cells [39]. However, *Salmonella* can also kill infected DC by a mechanism dependent on the type III secretion system [40, 54]. Whether DC that have undergone *Salmonella*-mediated death are also a reservoir of cell debris containing *Salmonella* antigens that can be presented by neighboring, bystander DC is presently not known. Interestingly, the capacity to act as a bystander antigen presenting cell appears to be a unique feature of DC, as bystander macrophages ingest *Salmonella*-induced apoptotic cells but do not present peptides from *Salmonella* antigens [42]. Preliminary data suggest that macrophages compete for apoptotic material and limit the antigen available for presentation by bystander DC [42].

The precise nature of the material in the cell debris responsible for the observed bystander presentation of *Salmonella* antigens from apoptotic macrophages is not known. However, neither peptides released into the environment that bind preformed surface MHC molecules on bystander DC nor bacteria released into the surroundings that are subsequently phagocytosed and processed by the bystander cells account for the observed presentation [42]. Additional experiments are needed to characterize the antigenic material derived from the dying, *Salmonella*-infected cells.

Thus, DC can either be direct or indirect presenters of *Salmonella* antigens. They directly present bacterial antigens to T cells upon phagocytic processing of *Salmonella* that does not induce their death. They can also present bacterial antigens to T cells as bystander antigen presenting cells that engulf antigenic material from neighboring cells that have undergone *Salmonella*-induced apoptotic death.

35.4
Time to go to Work: Salmonella-induced Dendritic Cell Maturation

A steady-state population of DC is found in essentially all tissues of the body. DC situated in non-inflamed, non-infected tissues are in a so-called immature state, poised to respond to infection and capture antigen. Immature DC in peripheral tissues have a high capacity to internalize and process antigens, including bacteria, but a relatively poor capacity to stimulate naïve T cells [69, 70]. Immature DC express significant levels of surface MHC-II and MHC-I and low to intermediate levels of CD86, CD80, CD40 and CD54 (ICAM-I). They also are quiescent with respect to cytokine production. Upon receiving signals indicating inflammation and/or infection, immature DC undergo a series of phenotypic and functional changes and become mature, or activated, DC. This transition changes the DC from cells pro-

grammed for capturing and processing antigens into ones specialized in presenting antigens and activating T cells, particularly naïve T cells. To accomplish this, DC maturation includes transient stimulation of endocytic capacity followed by downregulation of antigen capture [71], optimizing MHC synthesis, trafficking and stability, and increasing co-stimulatory molecule expression [69, 70, 72–74]. The maturation process also involves enhancing cytokine secretion and altering chemokine responsiveness, the latter directing DC migration from infected tissues to draining lymphoid organs [69, 70, 75]. The net result of DC maturation is that antigen-laden DC with optimized capacity to activate antigen-specific T cells are located in secondary lymphoid tissues. This underscores the importance of DC in initiating adaptive immunity.

As microbial compounds that signal through Toll-like receptors, such as LPS, and the cytokines IL-1β and TNF-α are well established inducers of DC maturation [69, 70], it is no surprise that *Salmonella* contact with DC initiates maturation. For example, murine DC pulsed briefly with *Salmonella* have a decreased capacity to present antigens upon subsequent encounter with bacteria [19]. This is similar to the progressive reduction in endocytic capacity of DC after exposure to TLR ligands such as LPS [71, 76, 77]. Although the mechanism of down regulation of endocytosis in response to LPS has been eloquently studied [71, 76, 77], how *Salmonella* reduces presentation from a second exposure to bacteria remains to be investigated.

Co-culture of *Salmonella* with murine DC [11, 16, 28] or with human monocyte-derived DC [78] also up regulates CD86, CD80, MHC-I, MHC-II, CD40 and CD54. The up regulation of molecules occurs rapidly and on the entire population of $CD11c^{hi}$ cells. Up regulation of surface molecules does not require live bacteria or active bacterial internalization by the DC [16, 28]. This suggests that the presence of the bacteria in the culture was sufficient to trigger the population to increase expression of the molecules. With the exception of CD80, increased expression of the molecules examined was apparent within 8 hours after infection and was maximal within 24 hours [11, 16, 28]. Upregulation of CD80 had a slower kinetics and more stringent requirements. For instance, while upregulation of MHC molecules, CD86, CD40 and CD54 occurred in a similar fashion after a 2-h pulse with live or heat-killed bacteria, CD80 responded poorly [28] or not at all to heat-killed bacteria even when examined 48 h post infection [16].

Consistent with the induction of cytokines when DC are exposed to purified microbial ligands such as LPS, DC also activate NF-κB and produce several pro-inflammatory cytokines, as well as chemokines, in response to *Salmonella*. For instance, murine DC from the spleen, liver and cultured from bone marrow precursors produce TNF-α, IL-1β and IL-12p40 after a brief co-culture with *Salmonella* [7, 10, 13, 16, 28, 79]. However, despite robust IL-12p40 production by murine DC upon exposure to *Salmonella*, little IL-12p70 is detected [19]. This is in contrast to human monocyte-derived DC which produce IL-12p70 as well as TNF-α upon co-culture with *Salmonella* [78]. The inability to detect significant amounts of the IL-12p70 heterodimer in the murine system may reflect the absence of required augmentory signals such as CD40 engagement or IFNγ, or could reflect the presence of inhibitory cytokines such as IL-10 [80–82].

The ability of DC subsets to produce cytokines in response to *Salmonella,* as well as the relationship between bacterial uptake and cytokine production, have been studied by infecting DC with GFP-expressing *Salmonella* and assessing cytokine production by flow cytometry. These data showed that both $CD8\alpha^+$ and $CD8\alpha^-$ DC from the spleen and liver of mice produce IL-12p40 in response to *Salmonella*, and that the $CD8\alpha^+$ subset contained a somewhat higher fraction of IL-12p40$^+$ cells relative to $CD8\alpha^-$ DC [7, 10]. A higher fraction of splenic DC in the $CD8\alpha^-$ subset produce TNF-α compared to their $CD8\alpha^+$ counterparts while this trend was not apparent in liver DC, where the $CD8\alpha^+$ subset contained a slightly higher fraction of TNF-α-producing cells following co-culture with *Salmonella* [7, 10]. Production of IL-12p40 and TNF-α by DC did not require bacterial internalization but did require physical contact with the bacteria. Moreover, bacterial internalization did not necessarily result in production of either TNF-α or IL-12p40, as not all GFP$^+$ cells produced cytokine [7, 10].

These studies also showed that a significant fraction of cytokine positive cells were not associated with GFP-expressing bacteria [7, 10]. Indeed, the highest fraction of cytokine positive cells was among GFP$^-$ cells. Analysis of bacterial factors that induce cytokine production by DC showed that a diffusible product in the bacteria-DC culture could not induce cytokine production. This demonstrates that DC-bacteria contact is required, as mentioned above. Although TLR4-mediated signaling by LPS on *Salmonella* was not required for increased expression of MHC and co-stimulatory molecules by *Salmonella*-infected DC, LPS was involved in triggering cytokine production by DC [10, 13].Thus, LPS is an important bacterial ligand for cytokine production by DC, but other TLR ligands appear to be sufficient to induce up regulation of surface molecules in the absence of LPS.

Additional studies have addressed whether *Salmonella* with mutations resulting in LPS alterations influence DC maturation. For example, the effects of *Salmonella* with mutations in *phoP/phoQ* or *lpxM*, which have alterations in the lipid A portion of LPS relative to wild type bacteria [28, 83, 84] on down regulation of antigen presentation capacity, up regulation of co-stimulatory molecule expression and T-cell stimulatory capacity as well as cytokine production have been analyzed using murine DC [19, 28]. These studies showed that mutant bacteria expressing LPS with modified lipid A, or purified LPS containing a lipid A modification, had little if any alteration in their capacity to influence these aspects of DC maturation. However, given the observation that human but not murine TLR4 can discriminate between LPS containing lipid A modifications [85], it would be interesting to address the influence of lipid A modifications on maturation of human DC.

Although culture systems represent an environment not entirely similar to the milieu in an organ, particularly during infection where numerous cell types are activated and the cytokine environment is complex, they are valuable sources of information due to the capacity to manipulate parameters in a controlled fashion. However, *in vivo* infection models are an irreplaceable compliment to *in vitro* studies and have provided much insight into the role of DC and DC subsets during the course of *Salmonella* infection. Findings from murine infection models are summarized below.

35.5
Murine Infection Models to Study Dendritic Cell Interaction with *Salmonella in vivo*

35.5.1
Salmonella Infection and Penetration of the Intestinal Epithelium

Salmonella spp. cause a variety of diseases, from localized gastroenteritis to systemic illnesses. While *S. typhimurium* infection of humans remains localized and results in gastroenteritis, mice infected with this species get a systemic infection with pathogenesis resembling that of typhoid fever in humans, which is caused by *S. typhi*. Thus, a murine infection model is a useful tool to study systemic *Salmonella* infection.

Salmonella is transmitted by the oral route, and these bacteria must penetrate the intestinal epithelium to cause systemic illness. Data from murine infection models suggest that *Salmonella* may use more than one mechanism to cross the intestinal barrier. For example, penetration through M cells as well as via enterocytes in the epithelium overlying Peyer's patches can contribute to *Salmonella* penetration of the intestinal epithelium [86]. In addition, DC that breach the epithelial layer and sample the gut luminal bacteria also appear to have a role in transporting *Salmonella* across the epithelium [87–89]. Extending the original findings of Rescigno et al. [87], a recent report showed that lamina propria DC, particularly in the villi of the terminal ileum, extend dendrites between epithelial cells and are involved in the uptake of bacteria *in vivo* including orally acquired *Salmonella* [89]. When mice with lamina propria DC capable of forming transepithelial dendrites (CX_3CR1^+ mice) were infected with *Salmonella*, bacteria were recovered only from DC. In contrast, *Salmonella* are found in DC as well as in other phagocytes after oral infection of mice defective in CX_3CR1, which is required for DC to extend dendrites across the intestinal epithelium into the gut lumen [89]. The invasive nature of the bacteria may also influence the cell population(s) that harbor them after ingestion [88–90].

Evidence available thus far suggests that DC and/or other $CD18^+$ cells are responsible for initial bacterial internalization and dissemination of *Salmonella* in the infected host [87–92]. Additional studies showing the presence of $CD8\alpha^-CD11b^-$ DC in the follicle-associated epithelium overlying PP [5], which would be situated to sample intestinal bacteria, and that $CD11c^+$ DC in the subepithelial dome of PP contain microparticles that were orally administered to mice [93] further support a role of DC in mediating bacterial transit from the intestinal lumen. However, the presence of *Salmonella* in phagocytes other than DC ($CD11c^-$ cells) in the MLN and PP of orally challenged mice suggests that DC may not be the only cell involved in transporting invasive bacteria acquired orally [88, 89]. A caveat to this, however, is that the bacteria could have been internalized by other phagocytes in the MLN after transport to this organ in DC from the lamina propria or PP. Finally, although neither neutrophils nor macrophages appear to be resident populations in the subepithelial dome of PP [94, 95], they infiltrate PP and MLN in response to oral *Salmonella* infection (A. Rydström and M. J. Wick, unpublished

data). These cells could participate in microbial uptake in PP and MLN of infected mice, a possibility supported by the studies finding *Salmonella* in CD11c^{-} cells of MLN and PP after oral infection of mice with invasive bacteria [88, 89]. But whether the non-DC function mainly to kill bacteria or have additional functions such as shuttling bacteria in the host, presenting antigens to effector T cells, or providing bacterial antigens for bystander presentation by neighboring DC remain to be determined.

35.5.2
Dendritic Cell Take-up *Salmonella in vivo*

Murine models have shown that DC in the spleen, MLN, liver and PP harbor *Salmonella* during infection [7, 10, 36, 88, 89, 91]. A similar fraction of the three major splenic DC subsets (CD8α^{+}, CD8α^{-}CD4^{+} and CD8α^{-}CD4^{-}) contain *Salmonella* after administration of a given bacterial dose [10]. Likewise, roughly equal percentages of CD8α^{+} and CD8α^{-} DC in the liver of infected mice contain *Salmonella* after infection [7]. However, similar to the studies performed *in vitro* assessing bacterial uptake by freshly isolated splenic or liver DC discussed above, *in vivo* studies also showed that CD8α^{+} DC in the spleen had a higher capacity than the other two subsets to associate with bacteria at lower bacterial doses while at the highest dose tested CD8α^{-}CD4^{-} DC had the highest fraction of cells containing *Salmonella* [10]. Thus, the DC subsets may have differential access to bacteria depending on the bacterial load in the organ with CD8α^{+} DC possibly being better at scavenging a limiting number of bacteria.

Although DC internalize *Salmonella in vivo*, it is important to note that the number of DC containing *Salmonella* is very low, particularly in orally-infected mice. Whereas intravenous injection of *Salmonella* results in 1–10% of splenic CD11c^{hi} cells containing bacteria shortly after administration [10, 36], depending on the dose administered, splenic or liver DC associated with GFP-expressing *Salmonella* given orally are in such low numbers that their quantitation by flow cytometry is not reliable (our unpublished data; see also [7, 91]). In PP and MLN, however, quantitating cell-associated GFP^{+} *Salmonella* in the early stages of oral infection is possible [88, 91, 115]. In these organs, the number of DC containing *Salmonella* is ~5 bacteria/1000 DC in PP and ~1–2 bacteria/1000 DC in MLN [88, 115]. Cells other than conventional DC can also harbor bacteria after oral administration of invasive *Salmonella* as mentioned above [88, 115].

35.5.3
Getting the Game Started: Dendritic Cells Initiate Adaptive Immunity to *Salmonella*

35.5.3.1 *Salmonella*-induced Dendritic Cell Maturation During Infection

Total splenic CD11c^{+} cells respond to *Salmonella* infection by increasing surface expression of CD86, CD80 and CD40 beginning approximately one week after infection, depending on the bacterial dose and strain used [36, 115]. The appearance of DC exhibiting higher surface levels of co-stimulatory molecules corresponds to

the time post infection when *Salmonella*-specific T cells are beginning to appear [96]. Recent data also suggest that expression of these molecules occurs in a subset-specific fashion on splenic, MLN and PP DC in response to oral *Salmonella* infection. For example, CD80 is preferentially up regulated on CD8α^- DC in these organs, while the greatest increase in surface CD40 is on CD8α^+ DC and both CD8α^+ and CD8α^- up regulate CD86 [115]. Synchronous up regulation of co-stimulatory molecules occurs on the entire population of CD11c^{hi} cells in a given organ despite the low number of DC that contain bacteria. Moreover, up regulation of co-stimulatory molecules occurs simultaneously in PP, MLN and spleen despite the sequential seeding of these organs by orally administered bacteria [115]. These data suggest that indirect signaling by soluble mediator(s) rather than direct signaling by bacterial association/uptake are responsible for *Salmonella*-induced DC maturation *in vivo*.

In addition to the subset-specific differences in up regulation of co-stimulatory molecules, splenic DC subsets are differentially modulated with respect to number, distribution, and cytokine production in the early stages of oral infection [97]. For instance, splenic CD8α^+ and CD8α^-CD4$^-$ DC double in number five days after infection, while CD8α^-CD4$^+$ DC numbers are unchanged. The increase in CD8α^+ and CD8α^-CD4$^-$ DC was reflected by an influx of these subsets in the splenic red pulp of *Salmonella*-infected mice [97]. Quantitating changes in DC subsets in the MLN of infected mice revealed little change in the number of CD8α^+ and CD8α^- during the first five days of infection, while both subsets increased in this organ 2–3 weeks after infection in long term kinetic studies of mice infected with a strain of somewhat reduced virulence [115]. Thus, DC respond with quantitative changes in these organs in response to oral *Salmonella* infection.

An increase in CD8α^+ DC producing TNF-α, a cytokine critical for host survival to *Salmonella*, was also detected among splenic DC of mice orally infected with *Salmonella* [97]. However, the number of TNF-α^+ DC in the spleen during the first few days of infection is quite low relative to the number of TNF-α-producing neutrophils and macrophages in this organ [97, 98]. Given the relatively minor contribution DC appear to make to the overall TNF-α response during *Salmonella* infection, the function of DC-derived TNF-α comes into question. The low number of TNF-α-producing DC suggests that the major contribution of this population is not controlling bacterial replication through their bactericidal activity *per se*, a task likely attributed to the macrophages and neutrophils that respond to the infection [98]. However, TNF-α produced by DC during *Salmonella* infection may work locally in an autocrine or paracrine fashion to orchestrate DC maturation and migration. In this way, DC-derived TNF-α could facilitate linking innate and adaptive immunity.

In addition to TNF-α, IL-12 and IFNγ are also important for host survival to *Salmonella* [33, 99]. However, in contrast to the capacity of some microbial stimuli to elicit IL-12 by DC [5, 80, 81, 100–104], no significant increase in DC producing IL-12 was apparent in the first few days following oral infection [97]. Moreover, finding significant IL-12p70 in organ lysates or serum during the early stages of infection has been elusive despite finding increased IL-12p40 in infected mice (our unpublished data; [105]). These data would be consistent with a situation where oral-

ly acquired *Salmonella* induces a slow IL-12p70 response by relatively few cells. This could make detection of significant numbers of DC producing IL-12 difficult, and IL-12p70 may not readily accumulate to levels reliably detected in orally infected mice. The inability to detect significant IL-12$^+$ DC in *Salmonella*-infected mice may also reflect the limited numbers of bacteria in the spleen early during infection or the absence of additional signals necessary to enhance IL-12 production by DC in response to bacteria, such as those mediated through CD40 or by IFNγ [80, 81, 106]. It is also worth noting that even in systems where rather robust production of IL-12 by DC occurs in response to intravenous administration of microbial stimuli, the response is rapid and transient [80, 101, 102, 104, 107].

Thus, the "window" for finding IL-12p70, particularly if made by a nonabundant cell type such as DC, during oral *Salmonella* infection appears to be quite narrow. The lack of DC producing IL-12 does not seem to be due to production of IL-10 by these cells, however, [97], but it remains possible that IL-10 is produced by non-DC and reduces the capacity of DC to make IL-12 during infection [81]. Alternatively, the possibility that cells other than DC, such as macrophages and neutrophils, produce IL-12 during *Salmonella* infection requires further investigation [108, 109]. Thus, despite the role of IL-12 in host survival to *Salmonella* [110–112], the cell(s) responsible for its production, their relative contribution to the overall IL-12 response and the magnitude and kinetics of IL-12p70 production require further investigation.

35.5.3.2 Presentation of *Salmonella* Antigens by Dendritic Cells *in vivo*

As discussed above, DC in infected tissues (spleen, MLN, liver and PP) contain *Salmonella* during infection and respond by increasing co-stimulatory molecules and producing TNF-α. They are thus capable of interacting with T cells and participating in the adaptive immune response to this bacterium. Support for this comes from data showing that primary, antigen-specific CD4$^+$ and CD8$^+$ T cells are stimulated to proliferate upon co-culture with splenic DC isolated from *Salmonella*-infected mice, and that DC loaded with *S. typhimurium* can elicit bacteria-specific CD4$^+$ and CD8$^+$ T cells after transfer into naïve animals [10, 36].

Although these data indeed support a role for DC in stimulating *Salmonella*-specific T cells during infection, no additional information on, for example, the capacity of DC subsets in different organs to stimulate bacteria-specific CD4$^+$ or CD8$^+$ T cells during infection is presently available. Nor is there direct evidence that DC in infected animals are required to prime naïve bacteria-specific T cells, as has been shown for priming CD8$^+$ T cells during *Listeria* infection [113]. We are also lacking *in vivo* data addressing the relative role of direct presentation by *Salmonella*-infected DC to that of indirect presentation by non-infected bystander cells. The only thing that is known thus far is that both direct presentation of *Salmonella* antigens by infected DC and indirect presentation of *Salmonella* antigens by noninfected bystander DC can occur, and this data comes from *in vitro* systems (see section 35.3.2). No study has yet directly addressed the relative contribution of direct versus indirect presentation during *Salmonella* infection, as has been done for *Listeria*

[114]. In *Listeria*-infected mice, neutrophils were shown to be an important source of bacterial antigens that were crosspresented to $CD8^+$ T cells [114]. Similar studies assessing the capacity of cells that contain *Salmonella in vivo* but can not directly present antigens to T cells, such as epithelial cells and neutrophils, to be sources of bacterial antigens that can be crosspresented to $CD4^+$ and $CD8^+$ T cells by DC during infection certainly warrants investigation.

35.6 Concluding Remarks

Our knowledge concerning the role of DC and DC subsets in the immune response to *Salmonella* is advancing. However, there are many aspects of DC function during infection that remain to be elucidated. For example, little is known about the host and bacterial factors involved in DC activation and recruitment during infection. In addition, although recent studies have shed great insight into the cells used and mechanisms involved in transporting orally acquired *Salmonella* from the intestinal lumen to downstream lymphoid organs, this intriguing process is not yet fully understood. Elucidating the relative contribution of direct versus indirect presentation of bacterial antigens in activating $CD4^+$ and $CD8^+$ T cells during *Salmonella* infection, as well as the cellular reservoirs of bacterial antigens involved in indirect presentation *in vivo*, are also exciting aspects of anti-*Salmonella* immunity that need further study. Finally, determining the consequences of the differential response of DC subsets during *Salmonella* infection on adaptive immunity to this bacterium remains a challenge to tackle. Fortunately, studies using *Salmonella* infection models provide the exciting opportunity to address these issues and decipher the role of DC in the immune response to this pathogen in the complex cellular and cytokine milieu of infected lymphoid organs.

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36
Dendritic Cells in Tuberculosis

Ulrich E. Schaible and Florian Winau

36.1
Introduction

Tuberculosis (TB) is world-wide the most important bacterial infection in humans. The "white plague", as it was called in the 19th century, is part of the triad of most prevalent infectious diseases that also includes AIDS and malaria. The causative agent, *Mycobacterium (M.) tuberculosis*, was identified in 1882 by Robert Koch (1843–1910; Nobel prize 1905). However, the biology of the host–pathogen interaction is far from being conclusively elucidated, and current vaccination and therapeutic measures require significant improvement.

Dendritic cells (DC) are professional antigen-presenting cells (APC) with the potency to activate T cells through MHC class I, class II and CD1 molecules, which render DC indispensable for T-cell priming in TB. DC take up mycobacterial antigens for subsequent processing and T-cell activation [1]. Moreover, because they are able to phagocytose, DC engulf live mycobacteria and can thus serve as host cells for these intracellular pathogens. Here we discuss the multifaceted role of DC as APC in immunity against mycobacterial infection [2].

36.2
Tuberculosis

Tuberculosis is caused by *M. tuberculosis* and, in some African regions, *M. africanum*. *M. bovis*, primarily a pathogen of ruminants causing tuberculosis in cattle, can also infect humans, and the current live attenuated vaccine strain, *M. bovis* Bacille Calmette–Guerin (BCG) was derived from this species. *M. tuberculosis* is spread through aerosols from an infected individual by coughing. The mycobacteria primarily settle in lung tissue where they are engulfed by resident phagocytes such as alveolar macrophages and immature DC. Subsequently, infected cells migrate to the draining lung-associated lymph nodes (LAL) to mediate T-cell priming. Through DC, mycobacteria also reach the LAL but do not disseminate (Fig. 36.1) [1].

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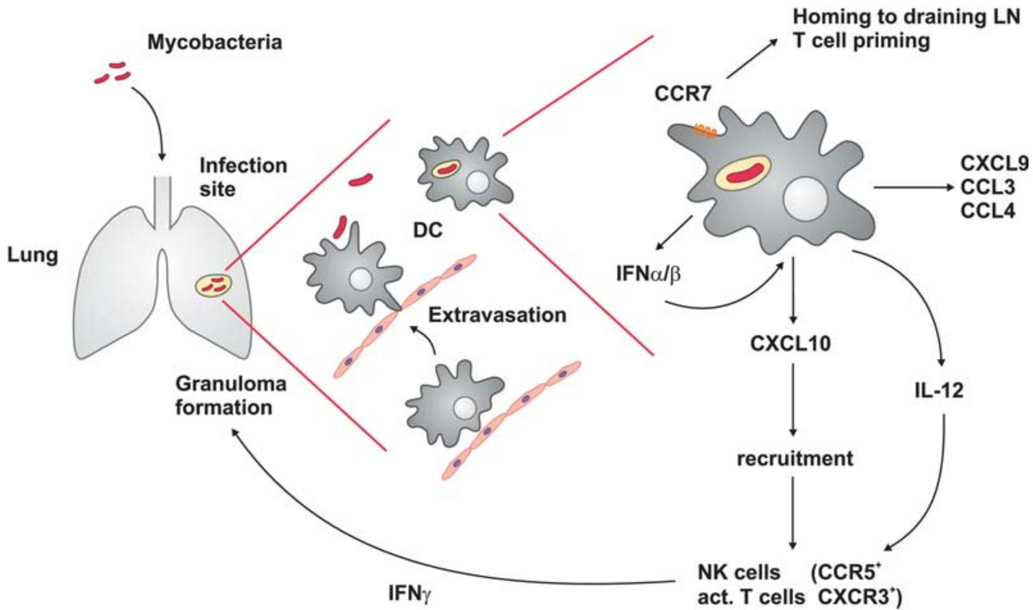

Fig. 36.1 Role of DC in tuberculosis. Inhalation of mycobacteria by aerosol leads to the infection of macrophages and DC in the lung. Infection activates DC to express CCR7 and to subsequently migrate to draining lymph nodes for T-cell priming. Concurrent release of type I IFN-α/β starts an autocrine activation loop initiating chemokine secretion (CXCL10, 9, CCL3, 4) to recruit activated T cells and NK cells to the site of infection. Onset of a protective T-cell response with IFNγ and TNFα as essential cytokines leads to macrophage/DC activation and granuloma formation to restrict spread of mycobacteria.

Cells of the innate immune system are activated by mycobacterial pathogen-associated molecular patterns (PAMP) through their pattern recognition receptors (PRR) such as the Toll-like receptors (TLR). Mycobacterial PAMP include lipoproteins, peptidoglycans, glycolipids, and low-methylated DNA (CpG). Subsequent chemo- and cytokine release by activated DC and macrophages finally recruits primed T cells and other lymphocytes to the site of infection [1]. In the immunocompetent host, the onset of the antimycobacterial T-cell response marks the limitation of infection within a histomorphological structure, the granuloma (tubercle). The granuloma is histologically characterized by concentric layers of immune cells. T and B cells surround an area consisting of macrophage-derived multinucleated Langhans giant and epitheloid cells harboring mycobacteria bordering a necrotic center [3, 4]. DC as defined by the expression of CD11c also promote granuloma formation but harbor less mycobacteria than macrophages [5, 6, 7]. DC from infected lungs, granuloma and LAL together are termed the Ghon complex. This restricted form represents the latent, clinically non-apparent stage of infection, which is estimated by the World Health Organization (WHO) to be present in one third of the world's human population. Latency at this stage of infection is due to a well-regulated protective immune response controlling mycobacterial growth and

spread. Less than 10% of infected individuals develop the disease [2]. The inner part of the granuloma liquefies and the containment of infection is abrogated. Subsequently, the mycobacteria grow unrestricted and spread hemo- and lymphogenically to other organs. Co-infection with HIV increases the risk of developing the disease 800-fold [2]. Apart from severe immunocompromising conditions, other reasons for the transition from the latent to the clinically apparent stage are not clear yet. Thus, in more than 90% of infected humans, immunity controls the infection. However, *M. tuberculosis* still kills 2 million patients annually world-wide [1]. The current vaccine in use, BCG, is only protective against systemic tuberculosis in children but not against lung infection in adults. The antibiotic therapy requires 6 to 12 months to be effective and depends on high compliance of patients [2]. Therefore, more efficient vaccination strategies and therapeutic protocols are needed to conquer tuberculosis in the future. Understanding the biology of the infectious agent and its survival strategies, as well as the pathways which lead to a strong protective immune response, i.e. antigen-processing/presentation and subsequent T-cell activation, are prerequisites to improve treatment. Thus, the biology of the DC as a central cell in host response to infection is an essential issue to unravel the pathogenesis of tuberculosis.

36.3 Mycobacteria are Intracellular Pathogens

M. tuberculosis and its relatives are facultative intracellular pathogens. Apart from those mycobacteria causing tuberculosis, other members of this genus have adopted a similar life style: *M. leprae,* the agent of leprosy; *M. marinum*, a fish pathogen sometimes causing skin ulcers in humans; *M. avium-intracellulare*-complex and *M. kansasii,* both opportunists mainly infecting immunocompromised patients. Their preferential host cells, macrophages, are primarily infected *in vivo* [8]. Moreover, mycobacteria also bind to receptors on DC, which facilitate entry into these cells. A C-type lectin molecule, DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), is a surface protein on DC. DC-SIGN is induced by IL-13 and IL-4 but negatively regulated by LPS and TNFα [9]. DC-SIGN binds mannose-residues and allows mycobacterial uptake by DC [10, 11]. However, other receptors have also been involved in this process such as the mannose receptor (MR), DEC205, scavenger receptors, and the complement receptor (C3R) [12, 13].

Upon uptake by macrophages, mycobacteria inhibit the maturation of phagosomes [14]. Thus, mycobacteria create their own intracellular niche with characteristics of an early endosomal compartment. The mycobacterial phagosome acidifies only mildly (pH 6.4) due to paucity of the vesicular H^+ATPase facilitating endosomal acidification. The early endosomal stage enables mycobacterial survival through access to iron, an essential growth factor. Iron is imported into early endosomes bound to transferrin (Tf) by the transferrin receptor (TfR). Tf/TfR intersects with the mycobacterial phagosome and delivers iron to the bacteria. Other marker-molecules which characterize the mycobacterial phagosome as an early endosomal

compartment comprise the small GTPase Rab5, and the vesicle fusion initiator molecule syntaxin 13 [14]. Mycobacterial phagosomes have only minute amounts of the lysosome-associated-membrane-protein-1 (LAMP-1) and lack late endosomal/lysosomal features, i.e. acidic pH, Rab7, active cathepsin D and the mannose-6-phosphate receptor. Mycobacterial trafficking inside DC is a controversial issue. One study suggests that mycobacteria grow as well in murine DC as in macrophages, whereas another report claims survival in DC but no growth [15, 16]. Experiments in the human system revealed that macrophages but not DC are permissive for *M. tuberculosis* [17]. Upon activation by IFNγ, which enables macrophages to kill mycobacteria efficiently, DC restricted mycobacterial growth but were unable to kill them. In DC, the compartment harboring mycobacteria appears to be different from the early endosome-type compartment in macrophages. Inhibition of phagosome maturation seems to be incomplete in DC since less than 50% of the phagosomes contain the early endosomal markers Tf, TfR and the antigen-presenting molecule CD1a [18]. An independent study demonstrated only limited presence of the early endosomal markers Tf and Rab11 (a recycling endosome specific GTPase) but also the lack of lysosomal features such as a low pH [17].

M. tuberculosis biases IFNα-induced differentiation of human monocytes towards $CD14^+$ macrophages instead of generation of DC [19]. This strategy probably provides more host cells favoring mycobacterial multiplication. Taken together, trafficking of mycobacteria within DC is guided by unique mechanisms when compared to macrophages.

Mycobacteria are covered by a rigid and hydrophobic cell wall consisting of an array of genus-specific glycolipids, glycophospholipids and waxes including 80-carbon-long fatty acids, the mycolates. This causes the acid-fastness of mycobacteria facilitating diagnostic staining (Ziehl–Neelsen stain) and makes dryness, organic solvents and disinfectants less harmful to mycobacteria then to other microbes. Through ester bonds, mycolates form arabinogalactan mycolates (AGM), trehalose dimycolate (TDM; Cord factor) and glucose monomycolate (GMM). Other compounds are the phosphatidylinositol-anchored lipoarabinomannans (LAM), phosphatidylinositol mannosides (PIM) forming an outer capsule-like layer as well as mycocerosates which are putatively involved in virulence [20]. Mycobacterial lipids are T-cell antigens presented by CD1 molecules [21]. Mycobacterial proteins including ESAT-6, CFP-10, Ag85 and the p19 lipoprotein have been characterized so far as T-cell antigens. The most potent T-cell antigens are either secreted or released proteins [2]. Some of these have recently been further tested successfully as novel vaccine candidates. Although mycobacteria reside in non-mature phagosomes, they release lipid and protein antigens, which enter the endosomal system of infected cells. Thus, antigens reach hydrolytic compartments where processing and loading onto MHC-II, CD1b, CD1c and CD1d molecules occurs [18, 22, 23].

36.4
Dendritic Cells Present Antigens in Tuberculosis

The immune response elicited by mycobacteria comprises a multitude of T-cell subpopulations: MHC class II-restricted CD4+ T helper cells, MHC class I-restricted CD8+ cytotoxic T cells, CD1-restricted T cells expressing αβ T-cell receptors (TCR), and T cells expressing a γδ TCR. T cells primarily participate in protection by IFNγ release to activate the antimicrobial effector mechanisms of macrophages. Moreover, CD8 T cells can kill infected cells and deliberate mycobacteria [1, 2]. Apart from human γδ T cells not requiring classical antigen presentation for activation, DC represent the prime APC in TB. DC express sufficient amounts of MHC-I and MHC-II as well as CD1 molecules on their surface. Additionally, potent T-cell priming requires co-stimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) as well as CD40 present on DC (Fig. 36.2).

BCG-pulsed DC present antigens and activate T cells from vaccinated donors [24]. Using CD11c as a distinctive marker for selection, DC have been isolated from infected lungs. Lung-derived DC were able to stimulate T cells despite expression of low levels of MHC-II and co-stimulatory molecules [5]. Due to the potent antigen-presenting function, DC have been used as vaccine carriers and natural adjuvants to protect against *M. tuberculosis* infection. Murine DC pulsed with either whole mycobacteria (either *M. tuberculosis* or BCG) or CD8 and CD4 T-cell epitopes from Ag85 protected mice against *M. tuberculosis* challenge by inducing mycobacteria-specific IFNγ-secreting CD4 and CD8 T cells [25, 26, 27]. Co-stimulation through CD40 prior to vaccination further enhanced the capacity of these DC to stimulate protective immunity [28].

Dendritic cells are the only cells in humans expressing all 5 CD1 molecules. Group I CD1 molecules comprise CD1a, CD1b, CD1c and CD1e, and group II consists of CD1d. CD1 molecules are homologues to MHC-I molecules and also associate with β2-microglobulin, but exhibit only minor polymorphism [21]. CD1a is primarily expressed on Langerhans cells, a DC subtype within epithelia, and to some extent on immature and mature DC. CD1a is a residential protein of the early/recycling endosome pathway involving the GTPase ARF6. Therefore, CD1a does not require an acidified compartment for antigen acquisition. CD1b and CD1c are present on immature and mature DC. However, the half-life of surface presence of CD1b and CD1c is largely increased on mature DC, similarly to what is seen for MHC molecules. CD1b and CD1c traffic to late endosomes and lysosomes due to a tyrosine-containing endosomal-sorting sequence (YXXΘ). This sequence allows CD1b to interact with adaptor protein (AP) 3 for delivery to lysosomes [21, 29].

CD1 molecules present lipid antigens to T cells. The lipids identified as CD1 ligands are mainly derived from the mycobacterial cell wall [21, 29]: CD1a: sulfatides, lipopeptides [30]; CD1b: LAM, PIM, GMM, mycolic acid, sulfoglycolipid (such as Ac_2SGL) [31]; CD1c: isoprenoids; CD1d: PIM [32]. Most peripheral, CD8 T-cell specific for mycobacteria (BCG) in humans are restricted by group I CD1 molecules [33]. Freshly converted skin-test-positive donors indicating a recent contact with mycobacteria respond more frequently to CD1b– and CD1c-presented

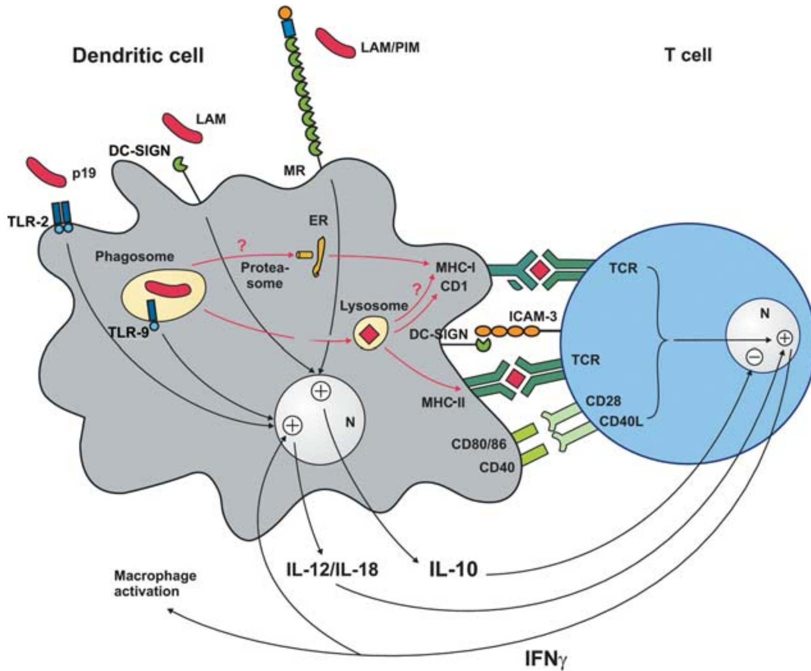

Fig. 36.2 The DC as immune-modulating APC. Mycobacteria and their pathogen associate molecular patterns (PAMP) such as lipoarabinomannan (LAM), the 19-kDa lipoprotein (p19) and low-methylated bacterial DNA (CpG) bind various pattern recognition receptors (PRR) on DC. Engagement of DC-SIGN and the mannose receptor (MR) induce release of the anti-inflammatory/immunosuppressive cytokine IL-10. In contrast, ligands for Toll-like receptors (p19 – TLR-2, CpG – TLR-9) activate DC to secrete the proinflammatory cytokines IL-12/IL-18. Thereby an IFNγ-dominated T-cell response (T helper type I) is initiated leading to macrophage activation and mycobactericidal effector mechanisms. DC are potent antigen-presenting cells employing MHC-I, -II, CD1 and co-stimulatory molecules (CD80, CD86, CD40) to prime mycobacterium-specific T cells. Mycobacterial antigens are delivered to the lysosomes for processing and binding to MHC-II and CD1 molecules. Loading of lipids onto CD1 molecules involves the lipid transfer proteins saposins. The pathway leading to MHC-I presentation within infected cells – whether through processing and loading within late endosomes/lysosomes, processing by proteasomes and loading in the ER, or by both – is yet unclear.

mycobacterial lipid antigens compared to skin-test-negative donors [31, 34, 35]. The question of enzymatic processing of lipid antigens within lysosomes remains unsolved. However, presentation of mycobacterial lipids by CD1b and CD1c requires transport through an acidified compartment. Loading of LAM, GMM as well as mycolic acid is facilitated through saposin C, a lysosomal lipid transfer protein (LTP) involved in lipid metabolism of mammalian cells [36]. Saposin C facilitates extraction of lipid antigens from intra-endosomal membranes and interacts with CD1b to allow lipid loading. Thus, the hydrophilic gap between membranes and the hydrophobic antigen-binding groove of CD1b is bridged by saposins.

CD1e is exclusively found intracellularly with a distinctive stage-dependent localization within the Golgi apparatus of immature DC and in late endosomal/lysosomal compartments of mature DC [37, 38]. The presence of CD1e only in DC suggests a specific function in antigen-presentation and lipid transfer. Indeed, CD1e binds glycolipids, and its soluble form is required for processing of high-mannosylated PIM_6 by lysosomal α-mannosidase to generate PIM_2 for recognition by CD1b-restricted T cells [82].

CD1d restricts natural killer (NK) T cells expressing NK1.1. CD1d has a broader cellular distribution including macrophages, certain B cells and epithelial cells, but is also expressed on DC [21]. CD1d also requires trafficking through acidified compartments for loading of exogenous ligands. However, CD1d also presents endogenous lipid antigens such as iGb3 (the lysosomal β-hexosaminidase degradation product of iGb4) [39]. Presentation of endogenous lipids and subsequent NKT cell activation requires induction of DC maturation through TLR engagement by PAMP [40, 41]. Purified mycobacterial PIM directly activates NKT cells in a CD1d-dependent manner in the absence of APC [32]. However, due to multiple PAMP present in mycobacteria, they could also activate APC to present self-lipids to NKT cells. Loading of CD1d with exogenous (as shown for the nominal NKT cell antigen α-galactosyl-ceramide derived from a marine sponge) and probably also endogenous lipids like iGb3 depends to some extent on LTP, mainly saposin A, C and the GM2 activator protein [42, 43].

DC express an array of surface PRR which serve as antigen-capturing receptors and/or signal transmitters. The TLR bind PAMP which in turn activate immature DC to migrate to draining lymphnodes and to express co-stimulatory molecules and cytokines. Langerin is a C-type lectin expressed by Langerhans cells. Langerin is localized in Birbeck granules, specialized early endosomal compartments carrying CD1a [44]. Birbeck granules probably function as antigen-loading compartments for CD1a since langerin facilitates lipid transfer to CD1a for T-cell activation. TLR present on DC and triggered by PAMP include (i) TLR-2 recognizing peptidoglycan, LAM and lipoproteins such as p19 lipoprotein [45, 46], and (ii) the endosomally located TLR-9 binding bacterial CpG. Interaction of mycobacterial ligands with TLR leads to the maturation of DC as shown by increased expression of CD83, CD80, CD86, CD54, CD58 and MHC-II [45]. This function of mycobacterial PAMP has been used to generate potent adjuvants for immunization as exemplified by the complete Freund's adjuvant and more sophisticated ones using liposomes containing purified PIM [47]. Further PRR on DC comprise DC-SIGN, DEC205 and the MR, which bind mannose-capped LAM and other mannose-containing mycobacterial lipids and proteins (see Section 36.3). Apart from binding and uptake of these ligands, these PRR appear also to mediate signals to the DC. Thus, interaction of mycobacteria with PRR activates DC and ultimately leads to the release of secreted mediators, i.e. cytokines and chemokines.

36.5 Dendritic Cells are Regulatory Cells in Tuberculosis

Upon interaction with mycobacteria, DC secrete cytokines and chemokines, which are important in determining the subsequent T-cell responses. Moreover, DC-derived chemokines play a pivotal role in tuberculosis by recruiting inflammatory cells to the site of infection and promoting tissue remodeling for granuloma formation. Mycobacteria induce DC to secrete cytokines such as IL-12, IL-18 and IFNα. Induction of IFNα is preceded by activation of an autocrine loop of IFNβ stimulation involving the transcription factors NF-kB and IRF-3 [48]. Type I IFNα and β subsequently activate DC to secrete the chemokine CXCL10 in order to attract NK cells and activated T cells expressing the respective receptors, CCR5 and CXCR3, to the site of infection [49]. CCR5-deficient mice control *M. tuberculosis* equally as wild-type mice but exhibit exacerbated pulmonary pathology [50]. This observation also points out that there is a certain redundancy with respect to the function of individual chemokines in tuberculosis.

Other chemokines induced in DC within the first 8 h of mycobacterial infection include CCL3, CCL4, CCL5 and CXCL9, which also participate in the recruitment and homing of activated effector T cells and NK cells [48, 49]. Mycobacteria also trigger DC to express CCR7, the chemokine receptor required for the homing of mature DC to draining lymphnodes in order to prime T cells [51]. Moreover, the chemokine receptor CCR2 is instrumental in recruiting DC but also macrophages and T cells to the mycobacteria-infected lung [52]. As a consequence, mice lacking CCR2 succumb much earlier to *M. tuberculosis* infection and show 100 times higher bacterial burden than wild-type mice. This suggests an additional defect in T-cell priming in the absence of CCR2. Moreover, migration of CCR2-KO DC to the infected lung is strongly delayed [53]. The importance of an early recruitment of DC to the infection site is supported by mathematical modeling of DC and T-cell turnover within the Ghon complex upon *M. tuberculosis* infection. This study points to the time range between infection and arrival of DC in the LAL and T-cell recruitment to the lung as a critical factor to tip the scales between latency and disease [54, 55].

Importantly, DC and NK cells establish a mutual relationship in tuberculosis. Upon mycobacterial infection, DC-derived IL-12 and IFNα stimulates NK cells as indicated by expression of the activation marker CD69, and enhance their cytolytic activity. In turn, NK cells trigger DC to mature and function as proper APC through signals involving direct cell-cell contact as well as TNFα and IFNγ secretion [56]. A similar reciprocal stimulation has been described for DC and γδ T cells involving DC-secreted IL-12 and T cell-derived IFNγ. This *pas de deux* however, does not require cell–cell contact [57].

Ligand-binding to DC-SIGN and MR induces the inhibitory cytokine IL-10 counteracting inflammatory responses induced by IL-12 [10, 11, 58, 59]. DC also produce IL-1-R antagonist, CCL22 and CCL17 but none of the pro-inflammatory cyto- and chemokines thus biasing the T helper 2 circuit [59]. Adoptive transfer of DC from IL-10 KO mice revealed that autocrine IL-10-production impairs both,

trafficking of DC to the draining lymph nodes and local IL-12 production [60]. However, mycobacterial stimulation of anti-inflammatory signals in DC must not only be seen as beneficial for the survival of the pathogen but is also of benefit to the host due to limiting inflammation-mediated pathology in tuberculosis lesions [61].

Engagement of TLR, mainly TLR-2, leads to the release of the pro-inflammatory cytokines IFNα, IL-1β, IL-12, IL-18 which biases towards IFNγ secretion by T helper 1, NKT- and γδ T cells as well as NK cells. IFNγ is essential for protective immunity in tuberculosis because it activates macrophages to optimally express their mycobactericidal effector mechanisms [1]. IFNγ induces the expression of inducible nitric oxide synthase (iNOS or NOS2) to generate toxic reactive nitrogen intermediates (RNI) and decreases TfR-expression to limit iron access to mycobacteria. In contrast to DC, mycobacteria-infected macrophages enter a different cytokine-expression program predominated by the pro-inflammatory cytokines IL-1, IL-6 and TNFα instead of IL-12. Macrophages also produce IL-10 suppressing IL-12-triggered T-helper 1 type responses [62]. A controversial report however showed that pro-inflammatory and immunosuppressive cytokines can be induced at the same time. *M. tuberculosis* induced IL-6 as well as IL-10 secretion in DC, which was mainly dependent on TLR-2 [63]. IL-10 converts DC into macrophage-like cells with increased capability of killing mycobacteria [64]. In contrast, TNFα-matured DC are even less able to control mycobacterial growth than immature DC [65]. Thus, DC and macrophages play distinct roles in the host response against mycobacteria: macrophages act first as pro-inflammatory cells and subsequently as mycobactericidal effector cells. DC however are primarily involved in priming and maintaining of antimycobacterial T-cell responses. Both cells are keeping an equilibrium between pro-inflammatory and anti-inflammatory responses to limit bacterial growth without overwhelming pathology [66].

36.6
Dendritic Cells and Cross-Priming

Live mycobacteria reside within early endosomal phagosomes. However, the phagosomal membrane secludes mycobacteria and their antigens from the cytosolic MHC-I pathway. Thus, activation of MHC-I-restricted CD8 T cells in tuberculosis remains elusive. Moreover, macrophages do not express group I CD1 molecules precluding CD1-restricted T-cell activation by primary infected cells. In addition, mycobacteria-infected macrophages as well as DC loose their antigen-presenting capacity quickly after infection [19, 67, 68].

Recently, the detour pathway in tuberculosis has been described involving apoptotic blebs from infected macrophages, which carry mycobacterial antigens to DC for subsequent CD8 T-cell activation (Fig. 36.3) [67, 69]. *M. tuberculosis* triggers apoptosis of infected macrophages, a process which has also been observed in tuberculosis lesions. Apoptosis of infected host cells leads to disintegration into and release of apoptotic blebs. Upon engulfment of these blebs, non-infected DC present mycobacterial antigens to CD8 and CD4 T cells through MHC-I, MHC-II

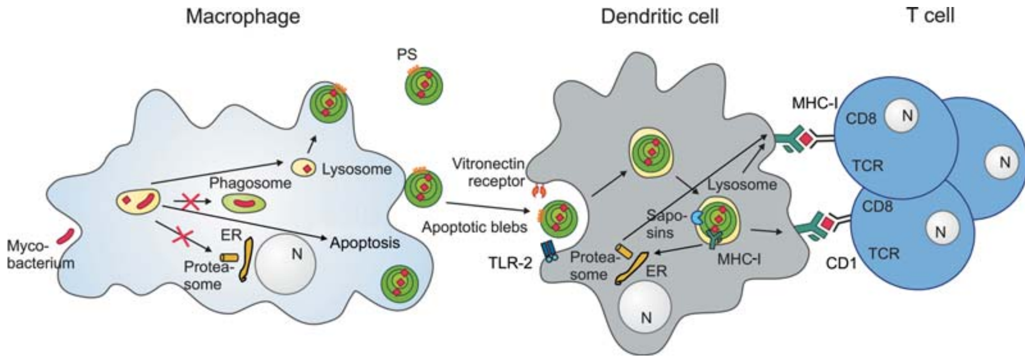

Fig. 36.3 The detour pathway of MHC-I and CD1 presentation. In macrophages, mycobacteria are segregated within phagosomes from the classical MHC class I pathway. Moreover, macrophages do not express group I CD1 molecules (CD1a, b, c). Finally, mycobacteria-infected cells loose their ability to present antigens to T cells. These hinderances to induce proper T-cell immunity are overcome by the detour pathway in tuberculosis. Infection-induced apoptosis leads to the release of phosphatidyl-serine (PS)-positive apoptotic blebs from infected cells. Thereby, mycobacterial antigens are carried to non-infected DC for presentation. Apoptotic blebs are engulfed by the vitronectin receptor (VR) and probably the PS-receptor and reach the endosomal system of the DC. DC maturation is initiated upon engagement of TLR-2 by mycobacterial PAMP (such as p19). DC subsequently prime T cells through MHC-I and CD1, but also MHC-II molecules. Processing of mycobacterial antigens is predominantly dependent on the lysosomal pathway. Saposins are involved in this process.

and CD1 molecules. Blebs from infected cells carry a triad consisting of antigens (proteins and lipids such as Ag85 and LAM), adjuvants (p19 lipoprotein and PIM) and antigen-presenting molecules (MHC-I) [70]. The adjuvant activates DC through TLR-2 [71]. Notably, the main adaptor molecule for the TLR signaling cascade, myeloid differentiation factor 88 (MyD88), is a prerequisite for cross-priming of CD8 T cells due to its function in DC maturation [72]. MHC-I molecules can be transferred by apoptotic blebs from macrophages to DC [70]. Thus, apoptotic blebs represent an autonomous immunological entity and mediate potent protection against tuberculosis upon vaccination in the mouse model [70]. In conclusion, host cell apoptosis represents an essential prerequisite for cross-priming of T cells in tuberculosis [69].

In DC, apoptotic blebs are targeted to lysosomes. Processing of the antigenic content for MHC-I presentation primarily depends on functionally intact lysosomes and less on proteasomal activity [68]. Saposins are instrumental in the processing of apoptotic blebs probably by disrupting the bleb membranes to release antigens [70]. The question as to how antigens subsequently intersect with MHC-I molecules in DC is not solved yet. However, a transport pathway for protein antigens from lysosomes to cytosol was described, which appears to be restricted to DC and absent from macrophages [73].

36.7 Mycobacteria Interfere with Antigen Presenting Cell Function

In tuberculosis, DC are essential APC and cross-priming appears to be a prerequisite for T-cell activation due to the interference of *M. tuberculosis* with APC functions [67]. Upon infection with mycobacteria, DC loose surface expression of MHC as well as CD1 molecules and become unable to activate T cells. The mycobacterial lipoproteins p19 and LprG (24 kDa) are responsible for interference with antigen presentation [74, 75, 76]. These lipoproteins are released from intracellular mycobacteria and shuttle to various compartments of the host cell including the ER. Engagement of TLR-2 by these lipoproteins causes down-regulation of MHC-II surface expression and activation of CD4 T cells in a MyD88-dependent manner. Moreover, mycobacterial lipoproteins inhibit MHC-I cross-presentation of phagosomal particle-associated antigens [77]. However, these studies have been conducted so far only in macrophage populations. Thus, the question is still to be asked whether mycobacterial lipoproteins have similar effects on DC.

Full maturation of DC is a prerequisite for potent induction of T-cell immunity. *M. tuberculosis*, even in the presence of strong triggers for DC maturation such as TNFα, IL-1β and prostaglandin E2 (PGE2), hampers maturation of human DC and consequently T-cell stimulation [78]. Moreover, upon infection of mice with ovalbumin-expressing BCG or listeria followed by the transfer of ovalbumin-specific T cells, antigen-specific activation of T cells was delayed and weaker in BCG-infected mice when compared to those infected with listeria [79]. *M. tuberculosis*-infected monocytes differentiate into CCR7- and CD83-positive DC upon GM-CSF/IL-4 stimulation, but express only low levels of CD1, MHC-II and CD80 molecules and fail to secrete IL-12. Consequently, these cells are unable to activate T cells *in vivo*. This has been correlated with the observation that in mycobacteria-infected mice DC were apoptotic for a prolonged period of time. Similarly, BCG hampers DC to develop their full APC potential, i.e. proper CD1, MHC-II, CD80 and CD40 expression [80]. These DC were unable to prime naïve T cells to undergo a T helper type I polarization, i.e IFNγ-secretion suggesting a putative reason for the ineffectiveness of BCG as a vaccine against *M. tuberculosis*. However, one study should be noted claiming that *M. tuberculosis*–derived secreted antigens initiate monocyte differentiation into DC in the absence of GM-CSF [81].

In conclusion, viable mycobacteria interfere with antigen-presentation through MHC-I, MHC-II and CD1 molecules there by hampening activation of CD4 and CD8 T cells. Inhibition of antigen presentation by mycobacteria renders the detour pathway of cross presentation through apoptotic blebs essential for cell priming. Activated mycobacteria-specific T cells can subsequently detect freshly infected cells before mycobacterial inhibition of antigen presentation occures.

36.8 Conclusion

DC are the main APC in tuberculosis to activate CD4 and CD8 T cells through antigen presentation by MHC-I, MHC-II and CD1 molecules. APC function however is targeted by mycobacterial virulence factors. This interference with T-cell activation is circumvented by cross priming through apoptotic blebs. Moreover, by releasing chemokines and cytokines, DC modulate subsequent T-cell responses and recruit immune cells to the site of infection. Thus, DC are central in the host response to mycobacteria, and unraveling their functions in protective immunity will allow better measures to control tuberculosis.

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37
Dendritic Cell–Epithelial Cell Interactions in Response to Intestinal Bacteria

Maria Rescigno

37.1
The Intestinal Epithelium and the Gut-associated Lymphoid Tissue (GALT)

The intestinal epithelium is the first line of defense towards dangerous microorganisms [1, 2]. It opposes a physical, electric and chemical barrier against luminal bacteria. The permeability of the barrier is regulated by the presence of both tight junctions (TJ) between epithelial cells (ECs) and a negatively charged mucous glycocalix. TJ seal adjacent ECs to one another and regulate solute and ion flux between cells [3]. The glycocalix sets the size of macromolecules that can reach the apical membrane of ECs [4] and opposes an electric barrier to bacteria. Finally, ECs and Paneth cells, specialized cells located at the base of the crypt of intestinal villi, release antimicrobial peptides including defensins and cathelicidins that target broad classes of microorganisms [5]. The intestinal epithelial barrier is further complicated by the presence of two important cell types that are interspersed between ECs and play a crucial role in sampling the luminal content: (microfold) M cells [6] and DCs [7–9]. M cells are found primarily in the follicle-associated epithelium (FAE) of Peyer's patches (PP) but they have also recently been described as being scattered among the absorptive epithelium, where they could potentially transport antigens to the lamina propria (LP) [10]. M cells, differently from ECs, do not have an organized brush border and are more permissive to antigen uptake [4]. DCs are phagocytic cells that are scattered throughout the intestinal epithelium [11]. We have recently reported that DCs are able to send dendrites out like periscopes into the lumen for bacterial uptake [12, 13]. The integrity of the epithelial barrier is preserved because DCs express TJ proteins and can establish new TJ-like structure with adjacent ECs [12]. These 'creeping' DCs are characterized by the expression of the myeloid marker CD11b and the lack of CD8α [13, 14]. Their presence in the terminal ileum, where the gradient of bacteria gradually increases, suggests they may be recruited by the presence of luminal bacteria. Interestingly, DCs in CX3CL1 (fractalkine) receptor-deficient mice are unable to spread their dendrites across the epithelial barrier, indicating the involvement of CX3CL1 in driv-

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ing the extension of the dendrites [14]. It is not known whether bacteria can directly drive fractalkine production by epithelial cells nor whether fractalkine modulates TJ protein expression in DCs. Interestingly, bacteria lacking LPS are unable to recruit DCs in *in-vitro* generated epithelial cell monolayers suggesting that bacteria play an active role in the induction of DC migration across the epithelial barrier [15].

The GALT can be divided into inductive sites where the immune response is initiated and effector sites where immune cells carry out their function [2, 16]. PP, mesenteric lymph nodes (MLN) and isolated lymphoid follicles are important inductive sites for mucosal immune responses whereas the epithelium and the lamina propria of the mucosa are considered effector sites for antibody production and T-cell responses.

37.2
Antigen Uptake in the Gut and DC Populations

Antigen uptake in the gut depends on the nature of the antigen. In fact, soluble antigens like digested food can penetrate through the meshes of the glycocalix and can be internalized by ECs throughout the intestinal wall. However, because absorptive epithelial cells rapidly degrade ingested proteins, it is likely that additional mechanisms of antigen uptake like the DC-mediated mechanism [12] are important in the mucosa. After internalization of soluble antigen either through ECs or through LP-DCs, the latter are probably involved in the induction of oral tolerance. In fact expansion of DCs *in vivo* enhanced tolerance induction after antigen feeding [17]. It is possible that antigen-loaded DCs migrate to MLN which is the preferential site for naïve T-cell activation and expansion after oral feeding of soluble antigen [18]. Conversely, particulate antigen is most likely taken up in PP as mice lacking PP are perfectly competent to induce antibody response towards soluble but not towards particulate (microsphere) antigen [19].

The mechanisms of bacterial entrance depend on their pathogenicity (Fig. 37.1). Most of the pathogens have developed strategies to penetrate ECs or to facilitate M-cell invasion (for a review see [1]), whereas noninvasive bacteria can enter mucosal surfaces either through M cells or DCs. M cells can release their 'cargo' to underlying phagocytic cells, including DCs, that can migrate to the interfollicular region of PP for T- and B-cell interactions, whereas DCs that take up bacteria directly across mucosal surfaces are likely to migrate to MLN. Interestingly, MLN set the border for mucosal compartment avoiding systemic spread of commensal-loaded DCs [20]. Neither mechanism discriminates between invasive pathogenic and noninvasive commensal bacteria. An alternative mechanism for antigen entry across a mucosal surface that also targets DCs and could be used for bacterial internalization, has recently been described [21]. It is mediated by neonatal Fc receptors (FcRn) expressed by adult human (but not mouse) intestinal epithelial cells that transport IgG across the intestinal epithelial barrier, and after binding with cognate antigen in the intestinal lumen, recycles the immune complexes back to the

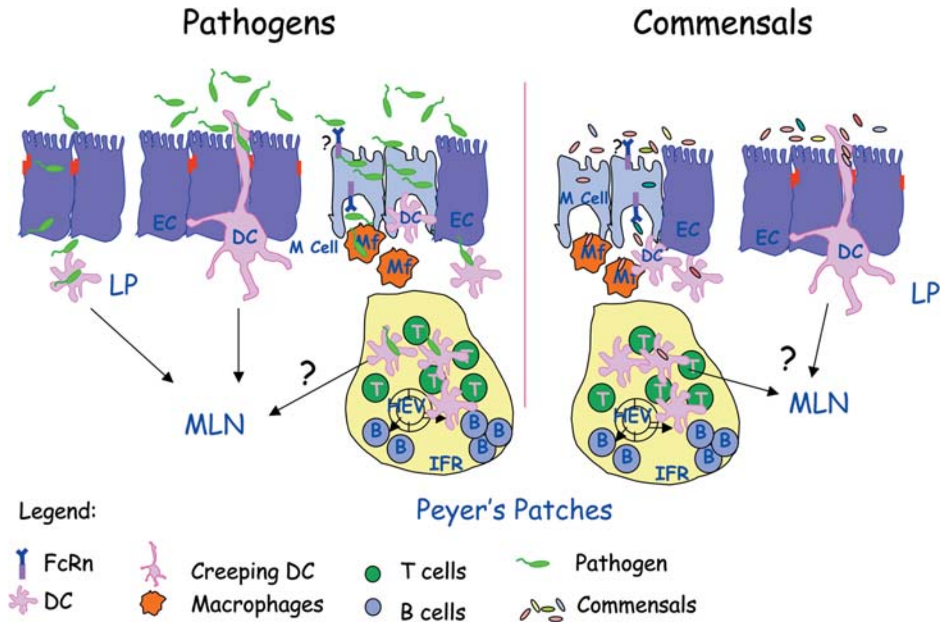

Fig. 37.1 Mechanisms of bacterial uptake. The mechanisms of bacterial entrance depend on their pathogenicity. Most of the pathogens have developed strategies to penetrate ECs or to facilitate M-cell invasion, alternatively they are captured by creeping DCs (left). Commensal bacteria can enter mucosal surfaces either through M cells or DCs (right). M cells can release their 'cargo' to underlying phagocytic cells, including DCs, that can migrate to the interfollicular region (IFR) of Peyer's Patches for T and B-cell interactions, whereas DCs that take up bacteria directly across mucosal surfaces are likely to migrate to MLN. Alternatively, PP-DCs could migrate to MLN. An alternative mechanism for antigen entry across a mucosal surface that also targets DCs and could be used for bacterial internalization is mediated by neonatal Fc receptors (FcRn) expressed by adult human (but not mouse) intestinal epithelial cells. FcRn transport directs and delivers the antigens in the form of immune complexes directly to underlying DCs. (HEV: high endothelial venules).

LP [21]. Antigens bound by IgG are less susceptible to degradation within the epithelial cells because endosomes formed after uptake by FcRn do not readily fuse with lysosomes. FcRn transport directs and delivers the antigens in the form of immune complexes directly to DCs lying in the LP. As DCs can be activated by immune complexes, it would be interesting to know whether DCs internalize the immune complexes via the FcγRs or via FcRn (both of which are expressed by DCs) and whether these receptors differentially affect DC function. Finally, DCs can process antigens from apoptotic intestinal epithelial cells, both in the steady state [22] and following reovirus infection [23], which constitutes another mechanism of DC antigen uptake that directly involves interactions with the epithelium.

The uptake route together with the nature of the ingested antigens dictates the type of immune response that is generated, whether this is related to the subtype

of DCs that is targeted by each route or to their location remains to be established. In fact at least four DC populations in the mouse intestine have been described. They are all characterized by the expression of CD11c but differ for the expression of the surface markers CD11b, CD8α and B220 (for a review see [8, 24]) as well as for the expression of chemokine receptors CCR6 and CCR7 [25]. Interestingly, the different DC populations have particular locations in PP [26]. In fact, it is important to say that in PP two important functions are carried out by DCs: uptake of antigen after its transcytosis across the FAE and T and B-cell activation. Therefore, differently from other peripheral tissues, it is possible to find in the PP both immature DCs that are mainly localized in the sub epithelial dome, below the FAE and mature DCs that are found in interfollicular T-cell areas. Two additional DC subsets have been described in MLN that are characterized by the differential expression of CD4 and DEC-205 [8, 24]. The characterization of human intestinal DCs is still very poor, but at least two DC cell types have been described in the colon: a CD11c+HLA-DR+ population and a CD11c– population [27] that we have identified as CD83+CD123+, possibly plasmacytoid DCs (our unpublished observations). Hence, scattered throughout mucosal tissues it is possible to find the same DC subsets present in other nonmucosal tissues.

37.3 Cross-talk between Bacteria and Epithelial Cells

The major interaction between mucosal tissues and luminal bacteria occurs at the level of ECs that are the most representative cell type of the epithelium. Both pathogens and commensal bacteria have been described to undertake an active cross-talk with ECs [1]. Whereas the first are primarily involved in the activation of an inflammatory cascade of events, the latter seem to downregulate the ability of ECs to initiate inflammatory responses. The mechanisms through which pathogens can activate ECs are similar to those used by monocytes and DCs to sense the presence of bacteria. In fact ECs express a series of pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs) and NOD proteins that are also expressed by phagocytes [1]. The major difference stands in the location of these receptors. In fact ECs seem to express these receptors either intracellularly (like TLR-4) or in a polarized fashion leaving the apical surface nearly free of PRR expression. Therefore only invasive bacteria or those equipped with type three or four secretion systems [28] that act as syringes to pump DNA or effector proteins directly into the cytoplasm of host cells, are sensed by PRRs for activation of the inflammatory cascade. Moreover, some of the receptors (like NOD2) are constitutively expressed only in Paneth cells [29] that reside at the base of the crypts and are induced in ECs only after bacterial encounter [30, 31]. A typical indicator of epithelial infection by invasive bacteria is the expression of the chemokine CXCL-8 (IL-8) which is a strong chemoattractant for neutrophils [32–35]. A more debated issue relates to the expression of TLR-5, the receptor for flagellin [36]. It has recently been described that flagellin-dependent stimulation of intestinal ECs results in triggering of

CCL20 via a TLR-5 dependent mechanism [37]. CCL20 is responsible for the recruitment of CCR6– expressing immature DCs [38]. However, some authors suggest that TLR-5 is expressed only basolaterally of ECs [39, 40], whereas others have described it also apically [37, 41]. We favor the second hypothesis because we have evidence that invasive-deficient mutant of *Salmonella* and the flagellated noninvasive soil bacterium *Bacillus subtilis* induce the expression of CCL-20 by polarized ECs [15, 42]. Our experiments in the mouse also confirm that noninvasive flagellated bacteria can induce the expression of CCL-20 suggesting the possibility that different responses might depend on the EC cell line used for *in vitro* experiments [42].

How commensals can downregulate the inflammatory response induced by pathogen associated molecular patterns (PAMPs) has only recently started to be unraveled. It is becoming clear that recognition of commensal flora via TLRs is required for intestinal homeostasis [43] and that commensal bacteria can interfere at different levels of TLR signaling. Expression and activation of IRAK-M [44] or of a truncated version of the TLR adaptor protein MyD88 [45] that both interfere with TLR signaling have been described. Along the same line, the interaction of ECs with the commensal *Bacteroides thetaiotamicron* or with nonvirulent mutants of *Salmonella typhimurium* interfere with the activation of NF-κB that is downstream of TLR signaling either by triggering binding of peroxisome-proliferator-activated receptor γ (PPAR-γ) with the NF-κB subunit Rel-A in the nucleus [46] or by blocking the degradation of IκBα, an intracellular inhibitor of NF-κB [47]. Therefore, the induction of an inflammatory response in ECs depends on the ability of invasive pathogens to activate PRR signaling pathways and on that of commensals to perturb the same signaling pathways.

37.4 Unique Functions of Mucosal DCs

DCs isolated from a variety of mucosal sites (PP, LP, mesenteric lymph nodes (MLN), lung) have the natural propensity to induce T_H2 responses in *in vitro* T-cell priming assays, and to express cytokines such as IL-10, and possibly TGF-β [2, 48–51]. Interestingly, the same $CD11c^+CD11b^+CD8\alpha^-$ DC subset isolated from PP but not from spleen preferentially polarizes antigen-specific T cells to produce T_H2 cytokines and IL-10 *in vitro* [52], suggesting that the observed differences are not attributable to subset-intrinsic properties but most likely to the local mucosal microenvironment. Further, the same PP but not spleen DC subset is able to promote IgA production by naïve B cells, which is mediated by a higher release of IL-6 [53] and T-cell help. These data suggest that mucosal DCs may be specialized in inducing a noninflammatory environment and in providing help to B cells via the activation of T_H2 T cells. This is consistent with the fact that many “tolerogenic” responses to mucosal antigens, for example to commensal organisms, are associated with the generation of antibody responses [20, 49], rather than with a broad immunological unresponsiveness. In addition, $CD8^+$ $CD11c^{lo}$ plasmacytoid DCs may also be

important for maintaining tolerance to innocuous antigens since this population can induce the differentiation of IL-10 producing regulatory T cells (Treg) *in vitro* [8].

Another important feature of DCs isolated from mucosal tissues is that they have the unique ability to selectively imprint gut-homing T cells [54–56]. Moreover, naïve $CD8^+$ T cells primed by PP-DCs acquire gut tropism [55], despite showing similar patterns of activation markers and effector activity as those primed by DCs isolated from other nonmucosal lymphoid organs. PP-DCs induced high expression of the intestinal homing integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9 in primed $CD8^+$ T cells. Interestingly, reactivation of skin-committed memory T cells with DCs isolated from gut changed T-cell tissue tropism, suggesting that memory T cells are relocated according to the tissue where they are needed [57]. Finally, mucosal DCs have been shown to continually migrate to draining lymph nodes in the "steady", or unperturbed state with a rapid turnover rate (2–4 days in the intestinal wall). In the rat, two types of migrating DCs could be identified, both of which are positive for the αE integrin CD103, but only the fraction that expresses low levels of CD172 (SIRPα), has features of immature cells and carries apoptotic enterocytes to MLNs [58]. Because these DCs process apoptotic epithelial cells in the steady state [22], this $CD103^+CD172^{lo}$ DC population may be involved in tolerance to self-proteins, although this hypothesis remains to be tested. DC emigration from the gut can be greatly enhanced by systemic LPS injection which does not change the proportion of SIRPα^{hi}/SIRPα^{lo} populations as well as their activation state [59]. Interestingly, whereas SIRPα^{lo} DCs migrate to T-cell areas of MLNs under steady-state conditions, SIRPα^{hi} DCs do so only after intravenous LPS injection suggesting that LPS injection facilitates antigen presentation by this DC subset.

37.5
Intestinal Immune Homeostasis is Regulated by the Cross-talk between ECs and DCs

DCs play an active role in bacterial uptake across mucosal surfaces and have unique functions that allow the generation of mucosal immune responses. Moreover, DCs can intercalate between ECs and can interact directly with the luminal bacteria and with all the TLR ligands that are carried by commensal or pathogenic bacteria. Therefore, three important questions arise: what is the role played by the local microenvironment in driving mucosal DC differentiation? How can DCs avoid the induction of exaggerated inflammatory responses towards commensal bacteria? Is there any relationship between the unique phenotype of mucosal DCs and the regulation of gut immune homeostasis? It is becoming clear that the relationship between DCs and the microenviroment profounds affects the functional properties of tissue DCs. This has been demonstrated in the spleen [60, 61], but there are strong evidences that a similar situation is occurring also in the gut. In fact, the ability of intestinal DCs to induce gut-tropism during T-cell priming [54–56] and reactivation [57] and to promote T_H2 T-cell responses [2, 48–52], as well

as IgA antibody production [53] strongly favors this hypothesis. As intestinal ECs are in close contact with DCs, they could play an active role in driving mucosal DC differentiation. We found that this is indeed the case and that ECs release constitutively TSLP, a molecule involved in driving T_H2 differentiation by DCs [62, 63]. Interestingly, DCs exposed to EC-conditioning are unable to release IL-12 and to drive T_H1 type of T-cell responses even after activation with T_H1-inducing pathogens (Fig. 37.2) [64]. Moreover, TSLP acts in a very narrow window of concentrations: at lower or higher TSLP concentrations, DCs reacquire the ability to release IL-12 and to drive T_H1 T-cell responses. Therefore, we believe that resident DCs

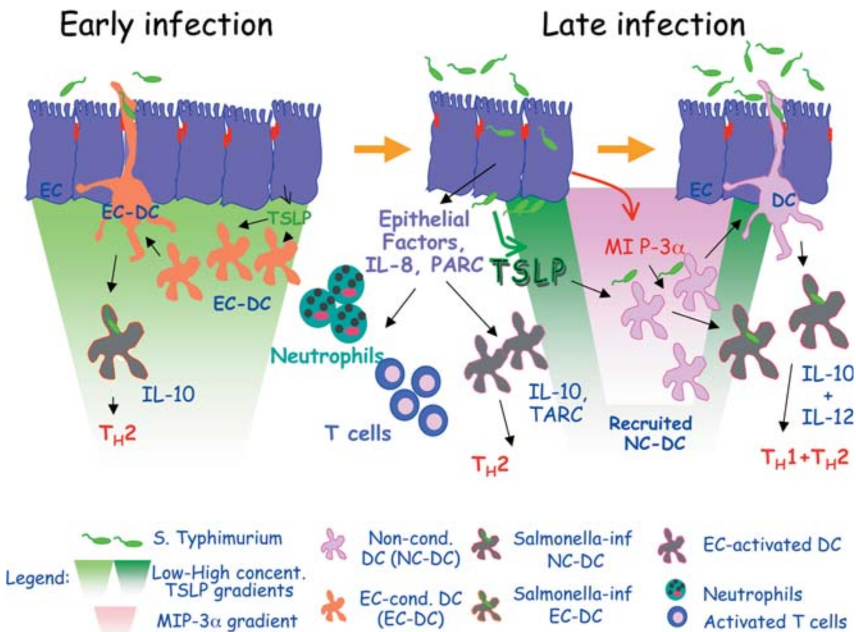

Fig. 37.2 Early *Salmonella typhimurium* infection: resident DCs are conditioned by EC-released TSLP (EC-DC). EC-DC release IL-10 after bacterial exposure and drive default T_H2 responses to *S. typhimurium*.
Late infection: since *S. typhimurium* is an invasive bacterium, it induces ECs to release pro-inflammatory chemokines like IL-8 (CXCL-8) and PARC (CCL-18), which attract neutrophils, granulocytes and activated T cells that generate an inflamed site. The binding of *Salmonella* to the basolateral membrane of ECs induces the upregulation of TSLP. TSLP at this concentration drives T_H1 rather than T_H2 promoting DCs in response to bacteria. Unidentified EC-derived factors can also activate 'bystander' DCs that have not been in contact directly with the bacteria. DCs activated in this way release IL-10 and TARC (CCL-17) but not IL-12, thus driving and recruiting T_H2 T cells. *Salmonella* also induces the release of MIP-3α (CCL-20) that recruits CCR6-expressing immature DCs.
Most likely, recruited DCs are not subjected to EC-conditioning, rather they could find increased TSLP concentrations in the infected site. Newly recruited DCs (NC-DC) can either creep between ECs to take up bacteria or they can phagocytose bacteria that have breached across the epithelial barrier and release both IL-10 and IL-12, thus promoting T_H1 and T_H2 responses. This allows the establishment of protective anti-*Salmonella* responses.

even though they have the chance to contact directly the bacteria, they are unable to activate inflammatory cells and this can help maintaining the homeostasis of the gut. In fact, nearly 70% of individuals affected by a T_H1-mediated chronic inflammatory disease like in Crohn's disease [65] have undetectable levels of TSLP and this correlates with the inability of intestinal ECs to regulate DC function [64]. Therefore, resident DCs that are actively involved in taking up bacteria at steady state do not drive inflammatory responses and this can explain why the intestinal immune homeostasis is preserved even though DCs are continuously exposed to TLR ligands.

37.6
Cross-talk between ECs and DCs in Bacterial Handling

Despite this propensity for the induction of T_H2 and Tregs by mucosal DCs, T_H1 and CTL responses are effectively generated to mucosal pathogens and are required to fight intracellular microorganisms [66–70]. Whether this involves the same or different DC subsets as those responsible for mucosal responses and tolerance induction, remains to be established. However, it is conceivable that resident mucosal DCs are 'educated' by ECs to initiate noninflammatory responses, whereas DCs recruited after bacterial invasion might retain their ability to respond in an inflammatory mode. In fact, infection by flagellated bacteria like *Salmonella* spp. induces the recruitment of DCs in the intestinal epithelium [12, 14] via the release of CCL-20 by ECs [37]. These nonconditioned newly recruited DCs might be responsible for the induction of T_H1 responses to invasive bacteria (Fig. 37.2). This hypothesis is supported by *in vitro* three-part studies in which DCs were seeded from the basolateral membrane of EC monolayers shortly before apical bacterial application [64]. Interestingly, due to their ability to creep between ECs and to contact bacteria directly, DCs were 'qualitatively' similarly activated regardless of the invasiveness or pathogenicity of the apical bacteria. Bacteria-activated DCs produced both IL-12 and IL-10 and skewed towards a T_H1 phenotype [64]. This suggests that nonconditioned DCs can drive the induction of inflammatory responses provided that they are not subject to EC conditioning before their encounter with bacteria. Moreover, bacteria invading ECs induce the upregulation of TSLP thus switching to DCs that have the propensity to induce T_H1- rather than T_H2-T cells in response to bacteria. Interestingly, bystander DCs that do not contact directly the bacteria are activated by EC-derived factors to noninflammatory DCs producing IL-10 and TARC (CCL-17) and inducing or recruiting T_H2 T cells, probably as a feedback mechanism to turn off the inflammatory response [42].

Another possibility is that epithelial cell derived factors, such as TNF or type 1 IFNs, produced during pathogen invasion may directly affect DC activation. This hypothesis is supported by studies of murine intestinal infection with type-1 reovirus [23]. Reovirus productively infects epithelial cells overlying PPs, yet viral antigen associated with apoptotic epithelial cells is avidly taken up by $CD11c^+$ $CD8\alpha^-$ $CD11b^-$ DCs in the subepithelial dome [23]. The observation that reovirus neither

productively infects DCs *in vivo* or *in vitro*, nor activates DCs to mature or produce cytokines *in vitro*, suggests a role for environmental factors, possibly derived from infected epithelial cells, in driving DCs to induce T_H1 responses to the virus. Interestingly, IFNαβR-deficient mice, but not MyD88-deficient or TLR3-deficient mice have an increased susceptibility to reovirus infection. In addition, MyD88-deficient mice mount normal IgG1, IgG2a/c and IgG2b responses, suggesting that type 1 IFN, possibly derived in the early stages of infection from infected epithelial cells, but not signaling via at least a single TLR pathway is important for inducing protection from reovirus infection.

Taken together, these studies highlight an important emerging relationship between DCs and epithelial cells in the maintenance of mucosal homeostasis and the induction of innate and adaptive immunity to mucosal infection with pathogens such as *Salmonella* and reovirus.

37.7 Conclusions

In conclusion, mucosal DCs have specialized functions that allow establish mucosal immune responses, including the induction of T_H2 T-cell responses and IgA antibody production. DCs play a crucial role both in the uptake of intestinal bacteria and in the induction of tolerance and immunity towards them. However, it is not yet fully clarified whether different DC subsets have clearly distinct functions *in vivo* or whether the local microenvironment is responsible to control DC function. Important issues that also need to be addressed are where DCs interact with T cells for the induction of regulatory or effector immune responses and if there are specialized induction sites that allow the generation of tolerance versus immunity, or systemic versus mucosal immune responses.

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XIII
Viruses

38
Sleeping with the Enemy: The Insidious Relationship between Dendritic Cells and Immunodeficiency Viruses

L. Vachot, S.G. Turville, S. Trapp, S. Peretti, G. Morrow, I. Frank and M. Pope

38.1
Introduction

38.1.1
The Global AIDS Epidemic

The epidemic caused by human immunodeficiency virus (HIV) continues to pose an immense global threat, particularly to the less well-developed countries. The 2004 UNAIDS/WHO Report on the Global Acquired Immunodeficiency Syndrome (AIDS) Epidemic (released December 2004) estimated that there were 4.9 million new HIV infections in 2004 bringing the total number of people (adults and children) living with HIV to 39.4 million by the end of 2004 (25 million of these people live in Sub-Saharan Africa). HIV-infected children totaled approximately 2.2 million and 17.6 million, of the 39.4 million HIV-infected individuals, were adult women (15–49 years old). Of the more than 20 million individuals who have succumbed to this disease since its identification in 1981, an estimated 3.1 million adults and children died from AIDS in 2004. Adding to this, an estimated 15 million children (from the ages of 0–17 years) had become AIDS orphans by the end of 2003 (3.5 million more than 2 years earlier).

There have been major advances in our understanding of HIV biology and in the treatment of HIV-infected people with antiretroviral drugs, to help control HIV infection and the development of AIDS. However, HIV infection continues to decimate entire communities, changing the whole structure of the society. HIV is most frequently acquired by sexual transmission across the mucosal surfaces (genital, rectal, and oral surfaces), but also via direct blood contact (intravenous drug use and

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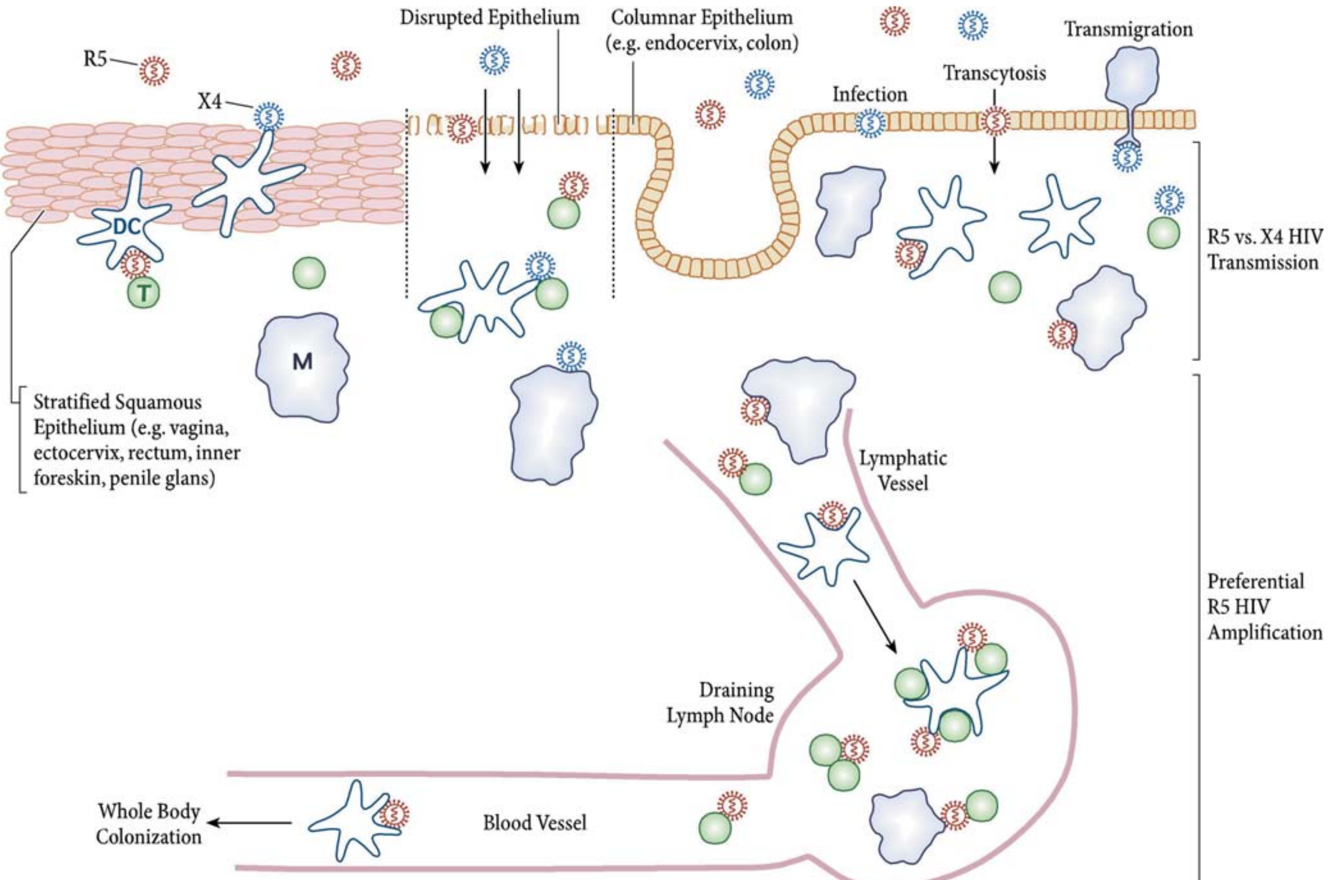
transfusions), and from mother-to-child (*in utero*, during birth, and breast feeding). To tackle these different routes of infection, multiple broad-acting approaches are imperative to most effectively limit HIV spread worldwide. Prophylactic and therapeutic vaccines could prevent new, and control established, infections, as well as limit the spread of infection between individuals [1–9]. To counter the dominant mode of sexual transmission, increasing emphasis is also being placed on identifying products (e.g. microbicides) that can be applied topically to prevent mucosal infection [10, 11]. While research is advancing on both of these fronts, solutions are a way off and some basic questions remain unanswered about the biology of HIV transmission and the immune responses needed to control and/or prevent infection.

38.1.2 Overview of Dendritic Cell Involvement in the Onset and Spread of HIV Infection

An integral player in the initiation of HIV infection, but also in the induction of immune responses to HIV, is the dendritic cell (DC). We will discuss how immunodeficiency viruses exploit the DC system to favor the establishment of infection, while coincidently subverting the induction of potent antiviral immunity to further facilitate the onset of infection. This overview will cover the current knowledge on the interactions of HIV with different DC subsets and highlight how understanding the complexities of DC–HIV biology is critical towards the development of improved microbicide and vaccine approaches to limit HIV dissemination.

Being positioned at the body surfaces, as well as circulating throughout the blood and lymphoid tissues, DCs are in prime locations to interact with incoming pathogens, including HIV. In fact, Langerhans cells (LCs) within the outer epithelial tissues of the genital, rectal/anal, and oral mucosae, may be one of the first leukocytes to encounter HIV as it breaches the mucosal surfaces. However, the exact route via which HIV crosses the epithelial surfaces, in order to reach the target leukocytes, may be influenced by the type of epithelia (e.g. single columnar versus multilayered squamous) (Fig. 38.1) [12]. Cell-free and/or cell-associated virus could

Fig. 38.1 Potential cellular targets during mucosal transmission of HIV. At mucosal surfaces, the epithelium represents the first line of defense for the body. HIV needs to cross this protective layer to access its target cells and initialize an infection. This event is dependent on the thickness and integrity of the tissue. Faced with a disrupted epithelium (breaks due to physical injury or infectious lesions caused by other pathogens) the virus has direct access to the subepithelial resident cells constituting a mixture of DCs (DC), T cells (T), and macrophages (M), which is a favorable environment for local viral amplification. In the presence of an epithelial monolayer, the virus has several possibilities to reach the subepithelial compartment. HIV can either infect, transcytose, or pass between the epithelial cells. Similar events may happen in the presence of a multilayered epithelium, but in that case the probability for the virus to cross the entire layer is reduced. LCs located in the epithelial cell network may "trap" the virus using their extensions and conduct it directly to the subepithelial compartment. Once bearing the virus (as infected cells or cells with internalized virus; Fig. 38.3), DCs can migrate to the afferent lymph nodes where the virus is transmitted to the T cells, facilitating systemic virus dissemination. While both R5 and X4 viruses possibly cross the barrier, there appears to be preferential amplification and dissemination of R5 viruses.


R5
X4
DC
T
M
Disrupted Epithelium
Columnar Epithelium
(e.g. endocervix, colon)
Infection
Transcytosis
Transmigration
R5 vs. X4 HIV
Transmission
Stratified Squamous
Epithelium (e.g. vagina,
ectocervix, rectum, inner
foreskin, penile glans)
Lymphatic
Vessel
Preferential
R5 HIV
Amplification
Draining
Lymph Node
Whole Body
Colonization
Blood Vessel

(i) pass between epithelial cells (transmigration or trapping by DC extensions), (ii) infect epithelial cells, and/or (iii) transcytose epithelial cells to gain access to leukocytes within and beneath the outer layers [13–16]. Passage through tissue breaks caused by physical trauma or other infections (i.e. those causing ulcerative lesions like HSV-2) would facilitate direct access to (and potentially infection of) leukocytes within the epithelia (e.g. LCs) as well as to DCs, T cells, and macrophages in the underlying submucosa.

Efficient entrapment by DCs is believed to contribute significantly to the establishment of infection (Fig. 38.1). Viruses using CCR5 as their co-receptor (R5 HIV; macrophage-tropic) predominate early in infection [17–20] and can even encourage the recruitment of DCs [21, 22] to further enhance DC–virus interactions and DC-to-T-cell spread. Virus captured and internalized by immature and mature DCs, as well as virus newly produced by infected immature DCs, could be spread locally by being transferred from the DCs to submucosal T cells and possibly macrophages. Natural DC–T-cell communications would also promote virus growth within resting T cells that had captured virus directly [23, 24]. In addition, virus-carrying DCs probably migrate to the draining lymphoid tissues, where rapid spread of virus to resting and antigen-responding T cells [25] would occur in the constantly interacting DC–T-cell milieu. In fact, immature DCs reportedly accumulate in the lymph nodes during acute infection, providing additional targets for virus amplification [26]. All of these events would take place in parallel to the innate antiviral responses triggered by immunodeficiency viruses as well as the adaptive antiviral immunity, elicited by the virus-bearing DCs (below). But, as we will discuss, the virus ultimately supersedes the antiviral responses, allowing infection to establish and proceed, and the immune system to be destroyed. Understanding how HIV achieves this is central to identifying effective ways to block new, and to control established, infections through the use of microbicides, vaccines, and immune-boosting therapies.

38.1.3 *In vivo* Evidence for DC Involvement in HIV Infection

38.1.3.1 Macaque Studies on Mucosal DCs and Infection

The simian immunodeficiency virus (SIV)-macaque system represents an invaluable animal model to study the biology of HIV disease [27, 28]. This model allows tissue and blood to be sampled at precise times after infection via controlled exposure routes, as well as the testing of putative vaccine or microbicide strategies [10, 11]. Several studies have detailed the similarities between human and macaque DCs from blood and tissues [29–36]. Furthermore, macaque DCs interact with SIV much like their human counterparts *in vitro* [30, 37–39], emphasizing the value of the SIV-macaque system to study the role of DCs in HIV infection.

Early work identified the SIV DNA-positive cells within the vaginal epithelia as LCs or lamina propria DCs, that became infected following vaginal application of SIV [40]. Subsequent studies revealed that SIV RNA-positive LCs could be detected in the vaginal tissues within 1 day after exposure to SIV [41]. Additional reports ob-

served that virus-positive T cells were the first cells that were infected within 2–3 -days of mucosal exposure [42, 43]. This reflects direct T-cell infection and/or DC-to-T-cell spread, where extremely small amounts of virus within the DCs went undetected in the first days of infection. Together, this work supports the notion that DCs within the epithelial tissues are one of the first leukocytes interacting with incoming virus. Virus captured by these cells, as well as newly synthesized viruses coming from low-level infection of the DCs (below), is then transmitted to $CD4^+$ T cells that readily amplify infection within the mucosal tissues during the first days of viral exposure [44].

Research on chronically infected animals, demonstrated that the DC–T-cell milieu within the mucosal (and lymphoid) tissues was a significant site of virus replication [45, 46]. While virus-positive DCs were not prominent *per se*, clusters of DCs with virus-positive T cells (as well as some virus-positive DCs) in suspensions isolated from the tissues were apparent, underscoring the permissive nature of the *in vivo* DC–T-cell environment to infection. This supported earlier studies on human tonsils that demonstrated the presence of DC-containing virus-positive multinucleated syncytia within the DC–T cell-rich lymphoepithelia [47, 48]. Thus, in acute and chronic stages of infection, DCs create an obliging niche for HIV to propagate.

38.1.3.2 **Changes in DC Biology in Immunodeficiency Virus Infection**

Knowing that DCs might be targeted in HIV infection, there has been increasing interest in understanding how their biology is modified in HIV-infected individuals. Monocyte-derived DCs (moDCs) from HIV-infected people were shown to possess comparable functionality to moDCs from noninfected donors [49, 50]. As a result, subsequent studies using human [51–53] and macaque [31, 39] moDCs demonstrated that their ability to stimulate immunodeficiency virus-specific T-cell responses *in vitro* were intact. This suggested that the immunostimulatory capacity of DCs (generated from monocytes *in vitro*) remained unharmed during HIV/SIV infection. However, a study monitoring the function of highly enriched populations of infected moDCs indicated that HIV infection can dampen the immunostimulatory activity of DCs [54].

Advances in identifying (and isolating) the specific DC subsets in circulation (simplified here as $CD123^+$ plasmacytoid DCs, PDCs, and $CD11c^+$ myeloid DCs, MDCs) have revealed important details about DC biology in HIV infection that were previously not appreciated. PDC numbers in the blood of HIV-infected people decrease with HIV disease progression [55, 56]. This decrease in PDC numbers correlates with reduced type 1 interferon (IFN) levels [55, 57, 58] and increased opportunistic infections [57, 59] in HIV-infected persons. Some reports also describe reductions in the numbers and functions of both PDC and MDC subsets in blood with HIV disease advancement [55, 56, 60–63]. Recent macaque work similarly showed that characteristic PDCs and MDCs [33–36, 64] remained functional in healthy infected animals that did not yet show evidence of disease progression [36]. It is still to be determined whether reduced DC numbers will also result with progressive SIV infection and development of immunodeficiencies in macaques.

Moreover, comparable numbers of DCs were detected in the lymph nodes of healthy infected versus uninfected animals [65]. Whether the modifications in blood-derived DC numbers and function in advanced HIV reflects direct targeting and infection of the cells by virus [55, 66–70] or redistribution of cells to the lymphoid and/or other tissues [71] remains to be elucidated. The migratory responses of LCs and interdigitating DCs are altered in advanced SIV infection [72]. Infected DCs have been reported in the gut mucosa of SIV-infected macaques [73], but whether DC infection was initiated in the gut tissue or the infected DCs migrated to this site is uncertain. DC modifications in advanced HIV/SIV disease would impact the innate and adaptive responses in which DCs are integrally involved, thereby diminishing immune functions.

38.2 Consequences of DC–HIV Interplay

38.2.1 HIV-binding Receptors Expressed by DCs

Considering the DC system as two major subsets comprising myeloid-derived DCs (LCs, submucosal or dermal DCs, interdigitating DCs in the T-cell areas of the lymphoid tissues, germinal center DCs, MDCs in circulation, and *in vitro*-generated moDCs) and PDCs (in blood and lymphoid tissues), there is considerable diversity between these cells that influence how they interact with immunodeficiency viruses (Table 38.1). This diversity probably contributes to the initial DC–virus contact, the intracellular fate of virus, and how each DC subsets respond to virus.

With CCR5 being a major co-receptor for HIV entry into and infection of target cells [74, 75], the expression of CCR5 and CD4 by immature DC subsets represents one common way that these cells first interact with R5 HIV isolates [76, 77]. In more recent years, it has become apparent that DCs express several other receptors that can entrap viruses independent of their chemokine receptor (CCR) tropism. These include the C-type lectin receptors (CLRs) CD206 (mannose receptor), CD207 (Langerin), and CD209 (DC-SIGN) [78] which bind to the glycosylated sugars in the virus envelope proteins, thereby not discriminating between R5 and CXCR4-using (X4; T-cell-tropic) HIV isolates. Importantly, there are differences in the expression of the various CLRs amongst DC subsets (Table 38.1). LCs express CD207 but lack CD209 and CD206, moDCs and submucosal DCs express CD209 and CD206 [79]. PDCs are negative for CLRs that bind mannose-rich carbohydrates, but the data are controversial on whether PDCs and MDCs express CD209 [67, 79–82]. Of note, a small CD209$^+$ population is present in blood and represents 0.01% to 0.04% of the total blood leukocytes [83], but this subset co-expresses CD11b, CD14, and CD16 (lineage markers used for exclusion of monocytes and NK cells in currently defined PDC and MDC preparations).

In vitro studies demonstrated that HIV envelope (i) predominantly binds to immature moDCs, LCs, and dermal DCs via CLRs, (ii) CLR involvement is reduced

Tab. 38.1 HIV receptors expressed by distinct DC subsets and different stages of activation.

DC Family[2]	CD4 and CCR expression for infection					CLR expression for attachment		
	CD4	CCR5	CXCR4	Infection		CD206	CD207	CD209
LC				R5	X4			
ImLC	+[1]	+	+/-*	+	-*	-	+	-
MatLC	+	++	++	+	+	-	+	-
(MoLC)	+	+	+	+	+	+	+	+
Dermal DC								
ImCD14+	+	++	?	+	+/-	+	-	+
ImCD1a+	+	++	?	+	+/-	+	-	-
MatCD1a+	+	+	+	+	+/-	-	-	-
MatCD14+/-	+	+	+	+	+/-	+	-	-
MoDC								
ImMoDC	+	++	+/-	+	-*	+++	-	+++
MatMoDC	+	+	+	+/-	+/-	+	-	++
Blood DC								
MDC	+	+	+/-	+	+	-	-	-**
MDC (CD14+, CD11b+)	?	?	?	?	?	-	-	+
PDC	+	+	+/-	++	+	-	-	-
Mat Blood DC	+	++	++	-	-	-	-	-

[1] - No detectable expression/infection; + Low detectable expression/infection; +++ High detectable expression/infection; +/- Partial expression/infection; ++ Medium detectable expression/infection

[2] LC=Langerhan Cells, MoLC=Monocyte derived Langerhans like Cells, MoDC= Monocyte Derived Dendritic cells, MDC= Myeloid Blood Dendritic cells, PDC= Plasmacytoid Dendritic cells, Im=Immature, Mat=Mature.

* Conflicting observations/ Controversies

** Blood MDC populations with CD209 reported, although were later characterized as a separate MDC population with lineage markers CD14 and CD11b.

upon moDC and dermal DC maturation, and (iii) that PDCs and MDCs in blood and tonsils preferentially use CD4 to capture HIV [79]. DCs may also capture HIV via heparan sulfate proteoglycans (HSPGs) by envelope-independent, cholesterol-dependent mechanisms [84]. This may occur via the recently described exosomal uptake pathway in immature DCs [85]. HIV may also utilize other molecules like complement receptors, FcRs, and adhesion molecules acquired by the virus from the host cell (e.g. LFA-1/CD11a/CD18, ICAM-1/CD54, and ICAM-3/CD50) to interact with DCs [86]. The redundancy of receptors utilized by HIV to interact with different DC subsets emphasizes how HIV exploits DC biology to ensure its efficient capture by the cells. The receptors with which the viruses interact on the DC surface guide the fate of HIV in the DC (i.e. entry via fusion leading to infection or internalization of whole virions which might subsequently lead to infection or degradation).

38.2.2 HIV Infection of DCs

For several years following the initial demonstration that DCs capture HIV and transmit it to T cells [87] there was debate over whether DCs could be productively infected by HIV. It was not until larger numbers of DCs could be obtained through improved isolation and *in vitro* generation methods (i.e. from circulating monocytes or progenitors), that we could begin to fully appreciate the complex nature of DC–HIV biology. It is now clear that HIV can infect certain DCs, but also that the virus can be entrapped without necessarily leading to productive infection [88].

Much of the earliest work involved the use of matured DC populations that typically did not support robust virus replication if cultured in the absence of $CD4^+$ T cells [89]. However, being able to directly compare immature and mature *in vitro*-generated DCs revealed that the former could support productive infection with R5 HIV while mature DCs seemed quite resilient [90, 91]. Subsequent studies have confirmed that CCR5-expressing immature moDCs, LCs, MDCs, and PDCs can be productively infected with R5 HIV isolates [67–69, 76, 91]. Immature LCs became infected with R5 (but not X4) HIV following *in situ* exposure [76, 92]. As expected, immature DCs isolated from individuals carrying the Δ32 mutant in the CCR5 gene were unable to be infected by R5 HIV isolates [93, 94]. In addition, LCs isolated from Δ32/wild type heterozygous individuals were significantly less susceptible to HIV when compared with LCs isolated from CCR5 wild type individuals [92]. The fact that compounds targeting CCR5 impede infection also highlights the importance of this co-receptor for the infection of immature DCs (below).

Matured moDCs that express lower amounts of CCR5 are more resistant to infection (reviewed in [68]). Some data suggests that there is a post-entry block in the ability of mature DCs to replicate HIV [90, 91], with one study suggesting the inhibition occurs prior and post integration of the virus into the host DNA [91]. However, in light of more recent data, there are several possible reasons for the limited infection of mature DCs. As noted, mature DCs express lower levels of CCR5 and the attachment molecules and this has a significant impact on the overall fusion of virus with the cells, as well as altering the subsequent kinetics of virus-DC fusion [95]. Post fusion there is a delay in the appearance of full-length viral DNA transcripts in mature DCs; it takes 72 h in mature DCs compared to only 24–48 h in immature DCs [96]. Data from Bakri et al. [97] support the notion that the block is not at the level of reverse transcription or viral DNA integration, but rather that infection of mature DCs is limited primarily at the transcriptional level. This transcriptional block could not be attributed to altered expression and/or nuclear localization of NF-κB proteins or the SP1 and SP3 transcription factors [97]. HIV release also appears to be closely linked with the exosomal pathway in other antigen presenting cells (APCs) [98–101] and this pathway is downregulated during DC maturation [102]. Therefore, this might be an alternative explanation for the reduced viral production within mature DCs (compared to immature DCs in which this pathway is active and productive infection proceeds).

Although mentioned herein as productively infected, immature DCs are in fact infected at relatively low levels compared to lymphocytes and macrophages. Recent studies have reported that 0.08% to 4.77% of LCs are infected after being exposed to high titer virus *in situ*, comparable to moDC infection [92]. This observation does seem to be in contrast with the fact that immature moDCs are exquisitely capable of taking up large quantities of attached virus [38, 96, 103]. In addition, recent studies have also supported that this capturing of virus *en masse* by moDCs also results in significant levels of viral fusion when using R5 HIV isolates and consistently exceeds that observed within $CD4^+$ T cells [95]. Why there is so much fusion and (relatively) little subsequent infection with R5 isolates remains a paradox.

Virus attachment to moDCs is a major determinant of viral entry. This is illustrated by the fact that broad nonspecific CLR blocking with mannan or specific blocking with antibodies to CD209, along with the inhibition of complement-mediated attachment, all reduced the infection of immature DCs [79, 92, 104]. R5 HIV fusion with freshly isolated LCs and dermal DCs is as efficient as seen in moDCs, although it is unclear whether attachment plays a significant role in this process [95]. Kawamura et al. [92] observed that blocking with (currently available) CLR inhibitors does not effect the level of viral infection within LCs (whether more specific CLR inhibitors will affect LC infection remains to be determined).

With respect to X4 HIV entry, variable levels of CXCR4 may be present on the DC surface (Table 38.1). In $CXCR4^+$ DCs, only low levels of fusion occur [95, 103]. Thus, this limited fusion appears to be the principle factor underlying the observation of poor immature DC infection with such strains. X4 HIV fusion within immature LCs and dermal DCs isolated from skin, is observed at a higher level than what is observed in immature moDCs, although the values for R5 HIV are significantly higher in these DC subsets [95]. During maturation of moDCs, CD4 and CXCR4 are upregulated and subsequently results in increased levels of fusion with X4 isolates (although not near the range at which R5 isolates infect immature moDCs) [95].

Given viral infection of DCs in general is low and DCs do not need to be infected in order to transfer virus to $CD4^+$ T cells, why do we and others persist on studying the viral life cycle within them? This is largely because the ability of DCs to become infected, even at very low levels, translates into explosive virus replication once the DC encounters a $CD4^+$ T-cell [24, 76, 92, 105]. Recent studies have placed great emphasis on the ability of virus to be transmitted from DCs to $CD4^+$ T cells "*in trans*", and this process is independent of whether a DC becomes productively infected or not. Although this pathway may still occur *in vivo*, recent studies have observed that infection *in trans* is not selective for either X4 or R5 HIV isolates, has a half-life of 4 h, and is relatively inefficient at low and high viral doses [96, 105]. Although, other studies suggest that virus persists in a protected form within DCs for up to several days [106]. In contrast, DC infection, even at low-to-undetectable levels, results in selective viral transfer to T cells (R5>X4) [95, 96, 105, 107], which is consistent with *in vivo* findings of an HIV transmission bottleneck [18, 108]. The ability to transfer persists as long as the infected DC survives and is efficient even

when the DCs were exposed to low viral titers [105]. Recent studies by Popov et al. [109], have revealed that infected DCs can survive and produce virus for as long as 45 days.

Notably, DC infection, in the absence of CD4$^+$ T cells, produces viruses that acquire determinants from the DC membranes, while those virions produced in a DC-CD4$^+$ T-cell milieu express T-cell-derived determinants [110]. Viruses generated from different cellular sources (e.g. DCs, DC–T-cell mixtures, macrophages) may influence the subsequent spread of infection.

38.2.3 Internalization of HIV Particles by DCs

Due to the natural ability of DCs to capture and ingest particles, it is not surprising that a significant proportion of HIV is internalized. Internalization of virus occurs rapidly after virus binds to the DC surface [38, 88]. Once inside, immature DCs hold virus within small vesicles near the cell membrane while mature DCs position the viruses in larger vesicles deeper within the cell [38].

In the first minutes after internalization, virus appears to be in endosomal compartments [111], but within 30 min the virus moves out of the characteristic endo-lysosomal compartments [96]. These compartments do not co-stain for any of the classical markers (CD63, CD68, CD107a, CD107b, CD208, EEA1) [96] (IF and MP, unpublished observations), yet recent data confirmed that virus proteins do co-localize with the tetraspannin molecule CD81 [112] (IF and MP, unpublished observations). Some co-localization of virus with CD205 (DEC-205) in mature DCs has also been observed (IF and MP, unpublished observations). Such localization of virus within immature and mature DCs has been observed for different SIV isolates as well as both R5 and X4 HIV strains [38, 96, 113] (IF and MP, unpublished observations). Despite apparently escaping the classical endo-lysosomal pathway, internalized virus does get degraded by immature and mature DCs [96], with some of the virus also accessing the antigen processing machinery [39]. This has important implications for both DC-driven transmission of virus to T cells as well as the processing and presentation of virus for immune stimulation (below).

It is presumed that much of the virus internalization is mediated by CLRs like CD209 and CD206 since mannan can reduce the amount of virus captured by the DCs [96, 105, 114]. This is supported by observations that significant amounts of virus are still captured and internalized by DCs in the presence of agents that block CD4 (CD4-IgG2), CCRs (TAK799), or fusion events (T1249) [113] (IF and MP, unpublished observations), even though some of these blocking agents inhibited HIV infection of immature DCs [93, 115, 116]. CLR-virus interactions must be very brief and the CLRs recycled rapidly to the cell surface once the virus has been internalized, because there is limited co-staining of virus with either receptor within the cells [96]. Despite several reports, using transfected cell lines, suggesting that CD209 is the primary molecule involved is virus internalization, CD206 (not CD209) was significantly down regulated on DCs immediately following exposure

to HIV envelope [96]. CD206 has also been implicated in virus capture by macrophages [117]. Moreover, anti-CD209 antibodies rarely completely inhibit virus capture by DCs [96, 114]. Confusion also reigns when investigators attribute viral transfer to CD4$^+$ T cells to the capacity of the DC to internalize virus. Viral transfer can be dependent or independent on DC infection and in both cases they are (at best) only partially CD209-dependent [118].

38.3 DC-to-T-cell Transmission of Infectious Virus

38.3.1 Immunodeficiency Virus Replication in the DC–T Cell Milieu

The initial research on DC–HIV biology primarily demonstrated that DCs exposed to HIV [23, 24, 87] or SIV [30, 37, 66] promoted virus replication upon encountering CD4$^+$ T cells. This occurred effectively with activated T cells [87], but was also prevalent in co-cultures of virus-bearing DCs and resting memory [23, 24] as well as naïve [66] T cells. The extreme susceptibility of the DC–T-cell milieu to infection with immunodeficiency viruses was then supported by *in vivo* findings [45–48, 73, 119]. Even when resting T cells were loaded with virus *in vitro*, simply exposure to DCs could drive replication in this milieu [24]. Presumably within this cellular environment, the DCs signal the T cells sufficiently to allow replication within the T-cell pool, much like has been described within the mucosal lymphoid tissues *in vivo* where DCs and T cells would continuously interact [119–121]. CD40L signaling may trigger some of these events [122, 123], but the underlying molecular mechanisms still remain unclear.

HIV and SIV replicate in the DC–T-cell mixtures *in vitro* (reviewed in [68, 70]). Immature and mature DCs promote virus replication in concert with CD4$^+$ T cells [88]. In fact, very few contaminating T cells are sufficient to amplify replication in a DC-enriched culture. Studies comparing wild type pathogenic viruses to attenuated viruses that lack the *nef* gene revealed that this accessory protein is required for the replication of virus within the immature DC-resting T-cell milieu but not when mature DCs or activated T cells are present [124–127]. This suggests that Nef (alone or in collaboration with other viral determinants) can modulate the immature DC-resting T-cell environment to foster virus growth (below) and the absence of such might contribute to the attenuated replication of *nef*-defective viruses *in vivo* [128].

Within the Nef protein, the N terminus seems to be important for viral replication in T cells. The proline-rich motif (R71T) in Nef strongly modulates viral replication in primary T cells of immature DC–T-cell co-cultures [129]. Of the two naturally occurring Nef variants (R/T-Nef) that both downregulate CD4, only the R-Nef isoform supports viral replication [129]. This leads to the assumption that R-Nef interacts more easily with signaling pathways than the T-Nef does. Structural anal-

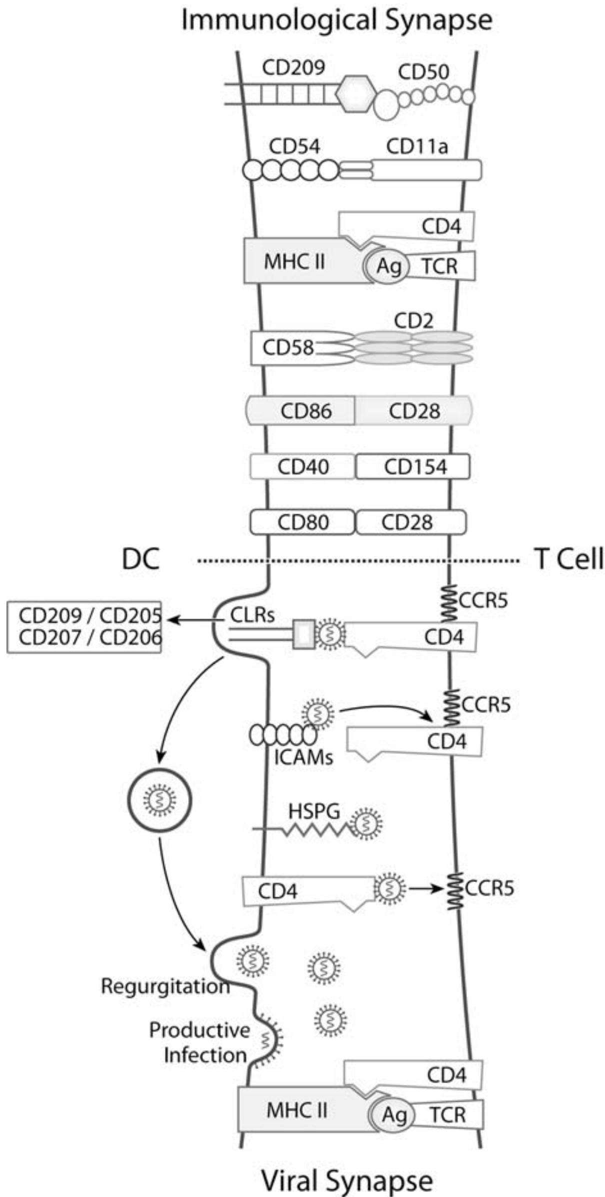
ysis suggested that the R to T conversion induces conformational changes, altering the flexibility of the loop containing the PxxP motif and, hence, its ability to bind cellular partners [129]. While Nef containing a mutation in the PxxP motif still induces chemokine release by immature DCs [125], PxxP mutant Nef-expressing DCs are less effective in stimulating autologous $CD4^+$ T cells to proliferate (compared to wild type Nef-expressing DCs; MP, unpublished observations). Secondly, Serine 6 in Nef has also an enormous impact on replication of HIV-1 in T-cell lines. It has been shown that a conformational change of Nef due to phosphorylation of Serine 6 by PKC θ/δ leads to a massive activation of the LTR activity (ST, unpublished observations). It is not clear yet how the PKC θ/δ is activated, but one can imagine that the communication between DCs and T cells is triggering this event [130–132]. Because $CD4^+$ T cells are so permissive to HIV replication, it is assumed that the bulk of the virus amplification occurs in the T cells within a DC–T-cell mixture, triggered by mechanisms like those described above.

38.3.2 Virus Movement across DC–T-cell Synapses

The first documentation of captured virions moving from a DC to a $CD4^+$ T-cell was achieved by video-microscopy carried out by McDonald et al. in 2003 [133]. Upon interactions of virus-loaded DCs with $CD4^+$ T cells, virus moved rapidly to the contact zone between the conjugated cells. CD4, CCR5, and CXCR4 molecules on the T cells accumulated at the point of contact, most probably enabling rapid movement of virus from the DC to the T-cell (Fig. 38.2). We similarly reported that virus proteins congregate at the contact sites between virus-bearing DCs and T cells and that increased numbers of virus-positive T cells could be detected over time [96]. This is true for both immature and mature DCs, despite immature DCs typically forming fewer conjugates with T cells.

Recent electron microscopic data has confirmed that whole virions accumulate in the tight contact zones between the conjugated (virus-bearing) DCs and T cells (IF and MP, unpublished observations). Therefore, internalized virions are simply regurgitated from the cells. Although virus protein-positive T cells were detected by immunofluorescent microscopy, we rarely observed virus particles bound to or being internalized by the T cells by electron microscopy (IF and MP, unpublished observations). This suggests that virus-T-cell fusion occurs rapidly upon release of the viruses from the DCs. Supporting this, inclusion of a fusion blocking peptide (T1249) [134] in the virus-loaded DC–T-cell mixtures, reduced the frequency of

Fig. 38.2 Events at the DC–T-cell synapse enabling DC-driven spread of virus to $CD4^+$ T cells. To facilitate DC-to-T-cell transmission, HIV uses the immunological synapse that occurs naturally between DCs and T cells during normal DC–T-cell communication. Thus, the immunological synapse becomes a viral synapse, across which the virus readily traffics. Many of the molecules involved in normal DC–T-cell interactions at the immunologic synapse are shown, compared to those exploited by HIV at the viral synapse. HIV can adhere to the DC using an envelope-specific binding with surface molecules (e.g.



CD4, CCRs, HSPG, or CLRs) or via envelope-independent binding. Host-derived proteins that are present on the viral particle promote the latter mechanism by binding their natural ligand on the cell surface (e.g. ICAMs). HIV particles at the DC surface can then be directly presented to the T cell. Alternatively, viruses internalized by DCs, as well as those newly synthesized by infected DCs, are released and handed over (as free virus or bound to a receptor on the DC) to the T cell.

virus-positive T cells. Interestingly, we did not observe an increase in the binding of virus to T cells in the presence of T1249 by electron microscopy, but rather a significant increase in the numbers of virus-carrying DCs, compared to what was observed in the absence of the inhibitor (IF and MP, unpublished observations). This probably represents the persistence within and/or re-capture of released virus particles by DCs, since virus cannot move over to fuse with the T-cell in the presence of the fusion blocker.

38.3.3
Two Phases of Virus Spread from DCs to T Cells

While the ability of DCs to amplify virus in collaboration with $CD4^+$ T cells has been well appreciated for several years, it was not until recently that two modes of transfer were definitively demonstrated and the kinetics dissected [96] (Fig. 38.3). Reflecting the rapid movement of DC-associated virus to the DC–T-cell synapse (above), the first phase of DC-to-T-cell spread was shown to involve the direct transfer of internalized particles. The ability of immature and mature moDCs to transmit entrapped virus diminished over 24 h, coincident with viral protein degradation within the DCs. Only in the case of immature DCs did we observe a rebound in virus transfer over time (second phase), when the R5 HIV (replicating at low levels in the immature DCs) was handed on to the T cells for further amplification. Inclusion of endosomal inhibitors to reduce virus degradation by the DCs, actually enhanced the infection of immature DCs, as well as their ability of immature DCs to transmit infection to T cells [107].

Recent work also suggests that these two phases of transmission are predominantly restricted to immature DCs exposed to R5 HIV isolates. Specifically, although immature and mature DCs can transmit R5 or X4 HIV to T cells during the first phase [23, 24, 91, 92], only immature DCs infected with R5 (not X4) HIV were able to mediate the second phase of infectious virus spread [96, 105, 107]. In fact, even when R5 HIV was undetectable in the DCs (as monitored using sensitive PCR techniques) a vigorous infection ensued once permissive T cells were added to the DCs [105]. This further supports the concept that DCs can capture and/or be infected by extremely small amounts of virus during the very first stages of mucosal infection. Although virus-positive DCs might be difficult to identify in some instances, such low levels of virus within the DCs is sufficient to subsequently amplify infection in the $CD4^+$ T cells within the local tissues (above).

More closely modeling *in vivo* events, Greenhead et al. [135] first reported that, while R5, X4, and dual-tropic R5X4 HIV isolates replicated in PHA-stimulated cervical explants, only R5 HIV isolates preferentially replicated if the tissues were left un-stimulated. Infection of the explants could be inhibited by agents blocking CCR5, CD4, or envelope, but not by the CLR blocker mannan [113]. In addition to infection of cells within the tissues, cells that migrated out of the explants were able to transmit infection to permissive cells. The ability of the migrated cells to transmit infection was impeded by the inclusion of CD4 and CLR inhibitors during initial exposure of the tissue to the virus, but not by CCR inhibitors. Therefore,

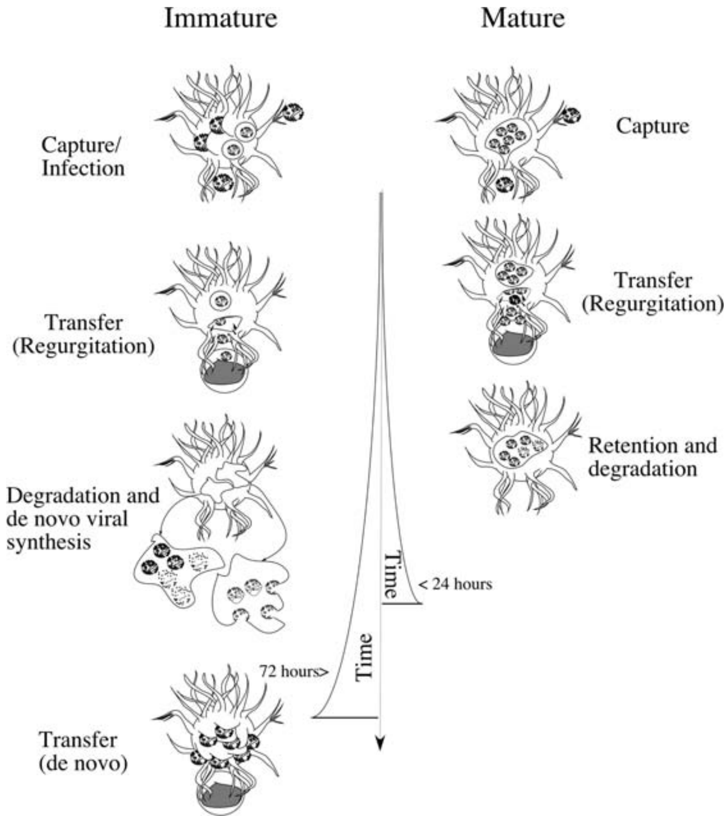


Fig. 38.3 Fates of virus captured by immature versus mature DCs. Depending on their maturation state, DCs will apprehend the virus in unique ways. Compared to mature DCs, immature DCs are more susceptible to HIV infection and can produce de novo particles (peaking at 72 h, although this may continue as long as the cell is alive). Moreover, the high adhesion abilities of immature DCs favor infection as well as particle internalization in small submembranous vesicles. In mature DCs, HIV is mainly captured and directed to large vesicles located deeply within the cell. Internalized virus within immature and mature DCs can be degraded or recycled for transfer (regurgitation) to other cells. The time period in which a mature DC bears infectious virus is a little longer than what is observed for immature DCs, but longer term transfer past 72 h only occurs in immature DCs as a result of de novo viral production.

CCR5/CD4-dependent infection and CD4/CLR-dependent capture mechanisms both contribute to the onset of infection of cervical explants. DCs and T cells within the tissues probably facilitate this and the bulk of the ability of the migrated cells to transmit infection resided in the HLA-DR$^+$ DC-containing fraction [113].

Recently, Chen et al. investigated the concept of nonrandom double infection in immature DCs [136]. When a mixed pool of virus was given to immature DCs, the DCs regurgitated virus in a manner to CD4$^+$ T cells that enabled double infection

at rates equal to (sometimes exceeding) that of cell-free virus. They also observed that two DCs carrying distinct homogenous HIV populations resulted in nonrandom double infection of the T cells, but at rates lower than that seen in cell-free viral challenges. The latter observation provides the first direct evidence that multiple DCs may be capable of binding and transmitting different viruses to the same $CD4^+$ T-cell.

38.4 Inhibiting DC-driven Infection

38.4.1 Preventing direct HIV Interactions with DCs and DC–T-Cell Mixtures

In the DC–T-cell milieu, productive infection mechanisms will dominate (since even DC-trapped virus will ultimately be handed over to infect the $CD4^+$ T cells or T cells will be infected directly by cell-free virus and infection driven by DC–T-cell communication). Broad-acting agents to prevent DC and T-cell infection, as well as capture of viruses by DCs, will be most effective in limiting DC-driven amplification of HIV. Blocking strategies impeding HIV envelope interactions with CD4 molecules or the CCRs have proven effective in limiting infections in DC–T-cell mixtures [66, 113, 115, 116, 137, 138]. As expected, productive infection of the DC–T-cell environment is also readily prevented by agents, such as dextran sulfate or carrageenans, that act through more nonspecific mechanisms to prevent virus-cell interactions (MP unpublished observations) [66].

When considering DC–virus interplay that occurs prior to the DC encountering a $CD4^+$ T-cell, the two separate pathways must be acknowledged [88] – capture and internalization versus infection. Even though immature DCs from individuals expressing the mutant CCR5 gene (Δ32 CCR5) were unable to be productively infected with R5 HIV [93], these cells still promoted at least low-level R5 HIV infection upon addition of activated T cells expressing the wild type CCR5 molecule [91]. More robust infection, comparable to that elicited by wild type CCR5-expressing DCs, resulted when the Δ32 CCR5 DCs were pulsed with an X4 HIV. Mannan or anti-CD209 antibodies do not completely block virus capture by DCs [84, 92, 96, 105, 113, 114], highlighting the potential involvement of non-CLR mechanisms. Inclusion of a blocking anti-CD4 antibody improves the inhibition of virus capture by DCs in the presence of mannan or anti-CD209 antibody, indicating some role for CD4. Even with the most effective anti-CD4/mannan combination 10–20% of the virus binding persists [96, 113]. In agreement, solely inhibiting CD4-envelope interactions with agents like the b12 antibody or CD4-IgG2, has little impact of the virus captured by DCs [113]. In contrast, CD4 and CCR5 inhibitors, but not CLR blockers, impede productive infection of LCs (and moDCs) [93, 115, 116, 139]. Even though neutralizing anti-envelope antibodies do not alter virus capture and internalization by DCs (above), moDC infection can be limited by the presence of the b12 or 2F5/2G12 antibodies [138].

38.4.2
DC-mediated HIV Transmission to T cells

HIV infection of T cells requires CD4 and CCRs [140–142]. Infection with viruses derived from DCs appears to involve a similar mechanism, independent of whether the virus is regurgitated from the DC or newly synthesized by the DC. Visualizing virus movement from the DC to the T-cell by video microscopy highlighted the participation of CD4 and CCRs on the T cells, that are recruited to the DC–T-cell synapse across which the virus traffics [133]. This observation helped explain earlier findings that molecules inhibiting virus envelope interactions with CD4 and CCRs effectively impaired transmission of infection.

Specifically, inclusion of neutralizing anti-envelope antibodies upon addition of virus-loaded human [138] or macaque [66] DCs to $CD4^+$ T cells prevented the amplification of virus in the DC–T-cell mixtures. In the case of the human DCs, this was shown to coincide with the impairment of DC infection [138]. More recent studies additionally showed that the viruses were still internalized in the presence of an anti-envelope antibody b12 or CD4-IgG2 but were unable to be transmitted to the T cells, most probably as a result of these agents remaining associated with the virus in the DCs and thereby blocking the subsequent T-cell-virus interactions needed for infection. Therefore, anti-envelope blocking agents can target both modes of DC-to-T-cell spread of virus. Furthermore, the ability of mature DCs to transfer virus to T cells for amplification was suppressed when various attachment, fusion, or entry inhibitors were added to the co-cultures [115, 116]. Even though CD209 contributes to virus capture by DCs, the transmission of virus from DCs to T cells appears to occur largely independent of CD209 [118].

Other bacterial and plant-derived agents binding HIV via glycosylation sites in gp120, like Cyanovirin (CNV) and the plant lectin UDA, were also recently found to very effectively limit DC-to-T-cell spread of virus. This was largely through carbohydrate-specific actions with HIV envelope that persisted once the virus had been endocytosed and, thus, later preventing virus-T-cell contact in a similar manner to that seen with the antibody b12 [105]. Blocking of viral transfer in the longer term, however, appears more elusive, as the efficiency of viral transfer from an infected DC greatly outscores that of a regurgitating DC that is not infected [105, 107]. Compounds that efficiently inhibit DC infection are obviously important, as only a few infected DCs need escape for a significant level of infection to proceed once in the presence of $CD4^+$ T cells [105]. In this setting, CNV and the CD4 down-modulating compound CADA, were observed to be the more efficient at blocking DC infection to undetectable levels when DCs were cultured alone, as well as when the DCs were placed in contact with $CD4^+$ T cells [105] (Kurt Vermeire, personal communication).

38.5 Functional Modification of DCs by HIV Favors Infection over Immunity

Beyond exploiting the variety of surface molecules expressed by DCs, HIV/SIV also manipulate the functional attributes of DCs in order to promote infection over the induction of effective antiviral immunity (reviewed in [68, 70, 143]). While DCs probably also mediate virus-specific innate and adaptive immune responses during the first stages of infection, these responses are insufficient to prevent or control the rapidly amplified and disseminated infection [44]. Amongst other possibilities, a general feature driving this is that immunodeficiency viruses do not activate DCs properly for them to function as potent APCs (unlike many other pathogens).

38.5.1 Viral Factors Modify moDCs

As we reviewed previously [70], HIV/SIV and/or viral determinants can directly modulate immature DC functions to promote virus growth. Nef is needed for virus replication in immature DC–T-cell mixtures but not when mature DCs are present [124, 126, 129]. Thus, Nef might be responsible for selectively modulating the immature DCs to behave more like a mature DC to drive virus replication while avoiding the induction of robust immunity. This limited replication of virus in an immature DC–T-cell milieu probably contributes to the attenuated replication of a *nef*-defective virus *in vivo* and the somewhat protective effects of this live-attenuated virus [128].

Support for this was provided by the observations that endogenously expressed Nef did not upregulate co-stimulatory molecules on immature DCs but did induce the release of chemokines and cytokines, increased clustering with lymphocytes, and elevated T-cell activation and virus growth [125, 127, 144–147]. Thus, in the absence of fully activated DCs, this milieu would elicit mediocre immunity (below) while encouraging leukocyte migration to the site of infection and cross-talk between the cells to foster infection. Nef-exposed DCs not only enhance DC interactions with $CD4^+$ T cells [148, 149], but also favor the induction of CD8 apoptosis and dysfunction [150], thereby further limiting effective immunity while driving infection. Even potential effects on innate DC responsiveness to pathogens might occur as a result of Nef down-modulating expression of CD206, as was recently reported for macrophages [151]. This appears to reflect a common selective effect of HIV on APCs [152, 153], since Nef similarly triggers macrophages to amplify infection in the macrophage–B-cell–T-cell milieu [154, 155].

Distinct from the effects of Nef on DCs, HIV Tat also modulates DC biology, inducing chemokine release to encourage T-cell and macrophage recruitment while avoiding the membrane phenotypic changes needed to mount effective immunity [156]. Another viral determinant, Vpr, has also been reported to downregulate co-stimulatory molecule expression upon addition to DCs *in vitro* [157]. Even HIV envelope protein reportedly interferes with the normal maturation responses of DCs *in vitro* [158]. Thus, several viral determinants either produced within the DCs or

by neighboring cells can exert modulatory effects on DCs that further encourage virus amplification and dissemination in the face of limited innate and adaptive immunity.

38.5.2
Effects of Virus on Circulating DC Subsets

The natural capacities of DCs in tackling pathogens probably also contribute to innate responses against HIV infection, that somewhat confine acute infection and control ongoing virus expansion in chronic stages. An emerging area of interest in innate anti-HIV immunity encompasses the potential involvement of defensins. Defensins may contribute to antiviral activities [159–163]. Myeloid-derived DCs secrete and respond to defensins [164–167], however it remains to be determined whether innate DC defensin responses contribute to restrict HIV infection. Innate cytokine and chemokine responses of myeloid-derived DCs, triggered by virus and/or viral determinants, probably contribute largely to facilitating virus amplification (above).

Innate responses of PDCs against viruses have been investigated more extensively, since PDCs were shown to produce significant amounts of type 1 IFNs in response to pathogenic stimuli [168, 169]. Importantly, it became apparent that HIV infection alters PDCs, since peripheral blood leukocytes exhibited reduced capacity to secrete type 1 IFNs and the numbers of PDCs decrease with HIV disease progression towards AIDS [55–58]. Studies examining blood PDC numbers in HIV-infected people receiving HAART revealed a negative correlation between PDC numbers and viral loads after cessation of treatment [170]. Additional work proved that PDCs actually produce large amounts of IFN-α in response to HIV [171–173] and SIV [36, 65], accounting for the lower type 1 IFN responses with advancing HIV disease when PDC numbers decrease. Notably, while PDCs secrete large amounts of IFN-α in response to inactivated HIV and SIV coincident with some bystander activation of the MDCs in culture [36, 172], the IL-12 responses were usually lower than that mediated by another DC stimuli, suggesting that the immunodeficiency virus-mediated activation was suboptimal. Additional activation of macaque PDCs with immunostimulatory oligodeoxyribonucleotides (ISS-ODNs) boosted the SIV-specific responses against MDC-PDC enriched mixtures presenting inactivated SIV *in vitro* [36]. This indicates that virus alone was not sufficient to fully activate the DCs to induce the strongest response.

PDCs within the lymphoid follicles associated with the mucosae are in prime locations to encounter incoming virions and may contribute to early events in HIV infection (infection versus innate immunity). Additionally, PDCs in lymphoid tissues (and blood) also contribute to anti-HIV responses throughout infection [174]. Work by Kier et al. [175] and Gurney et al. [176] highlighted that PDCs in thymic tissue are responsible for an IFN-α-dependent MHC class I upregulation in thymocytes, coincident with an IFN-α-dependent reduction in HIV replication in *ex vivo* tissue explants. Therefore, it is feasible that substandard type 1 IFN responses, as HIV/SIV infects PDCs [67–69], would also limit robust protection against virus

spread. Recent work in the macaque confirmed that PDCs within the blood [36] and lymph nodes [65] of healthy immunodeficiency virus-infected macaques (not progressing to disease) were still present in normal numbers and exhibited typical responsiveness to classical PDC stimuli. Whether PDCs in the lymphoid tissues also exhibit diminished functions with disease progression remains to be determined.

38.5.3
Virus-carrying Immature DCs Activate Substandard Virus-specific T-cell Responses

Research employing conformationally intact, noninfectious viruses inactivated with the chemical aldrithiol-2 (AT-2 viruses) confirmed that DCs can capture significant amounts of virus [38] and that at least some of this is processed and presented to stimulate virus-specific T cells [31, 39, 51, 177, 178]. Although immature DCs activated virus-specific $CD4^+$ T cells, mature DCs stimulated both $CD4^+$ and $CD8^+$ T-cell responses [39, 51]. This underscores how HIV/SIV can abuse immature DCs that they encounter during the earliest moments of mucosal transmission. By selectively stimulating $CD4^+$ T cells, this would intensify virus spread from immature DCs to the virus-specific as well as nonspecific T cells, in the absence of any attack from $CD8^+$ T cells. Even if the virus comes across mature DCs (e.g. in inflamed tissues) the mature DCs would spread virus to nearby $CD4^+$ T cells faster than most virus-specific T (or B) cell responses could be initiated [44]. In chronic infection, virus-bearing DCs would continue to promote virus growth. Additional virus-mediated events may restrict the induction of effective adaptive responses by varying DC-$CD4^+$ T-cell interactions to promote infection while limiting $CD8^+$ T-cell activation (above). HIV-exposed DCs can trigger $CD4^+$ T-cell apoptosis, further contributing to impaired T-cell responsiveness [179, 180]. Virus envelope expressed by infected immature DCs reportedly impaired the normal $CD4^+$ T-cell responses [54]. Moreover, infection of immature DCs prevented DC maturation coincident with eliciting IL-10 responses thereby hampering the induction of potent effector IFN-γ release [181]. Whether virus interactions with CLRs like CD209 trigger this immune dampening, as has been reported for *Mycobacteria* [182, 183], is not clear.

Further complicating the basic immature DC–HIV interplay is the potential impact of other pathogens on DC biology that can also lessen a DC's ability to elicit immunity. One example of this is *Herpes simplex* virus (HSV). Genital HSV (HSV-2) infection increases the likelihood of acquiring HIV infection [184]. This is probably due to herpetic lesions affording direct access of HIV to underlying mucosal and blood leukocytes, as well as to the HSV-induced suppression of DC immune function. Specifically, HSV infection of human DCs is cytopathic [185–187] and causes down-modulation of DC immunostimulatory function [188–190]. Exploring this in the macaque system, we recently verified that macaque DCs are susceptible to HSV-2 infection *in vitro* and that infection causes cytopathic and immunomodulatory effects in monkey DCs, as seen with human DCs [191]. Notably, we demonstrated that although HSV-2-infected immature macaque DCs captured SIV, they

induced poor SIV-specific T-cell responses *in vitro*. This provides direct evidence that a common infection like HSV-2 may compromise the DC system to further augment HIV spread. Understanding how such co-infections influence the ability of microbicides or vaccines to prevent HIV infection will be critical to optimize efficacy in limiting HIV transmission. The contrary is also true, that an HIV-modulated DC might favor the establishment of other infections and/or the loss of control of normally tolerated commensal organisms like *Candida* [192, 193]. Effective preventative strategies need to tackle HIV and co-pathogens (that might predispose one for HIV infection), boosting immune function to overcome virus-induced immune modifications.

38.6 Implications for Vaccine and Microbicide Strategies

38.6.1 Blocking Mucosal Infection

To press forward on studies performed using isolated DC subsets *in vitro* (above), increasing work has explored the use of tissue biopsies and even reconstructed mucosal tissues to study HIV transmission and its prevention through topical microbicide applications. Initial studies using skin epidermal sheets to mimic the mucosal surfaces confirmed that R5 HIV infections of LCs [76] could be inhibited with specific CCR5 inhibitors [77]. Work by Shi et al. [194], employed the use of Gene Gun technology to verify that attachment at the tissue/mucosal surface did not restrict infection of emigrating cells. Moreover, when a synthetic RANTES/CCL5 analog (a specific CCR5 ligand) was used to block R5 HIV infection of human immature LCs in a skin explant model, there was no evidence of increased propagation of X4 HIV [139]. These results further suggest how R5 HIV isolates have a competitive advantage over X4 HIV strains in establishing infection in LCs and reinforce the notion that replication with DC subsets at the periphery plays an important role in virus transmission (Fig. 38.1). HIV infection of LCs (derived from cord blood $CD34^+$ progenitors) within reconstructed vaginal mucosa was similarly blocked with CCR ligands [195].

Subsequently, R5 HIV infection of cervical explant tissues (comprising DCs and T cells) was shown to be inhibited by CD4- and CCR5-specific agents [113]. Notably, although not infecting cells within the tissue, virus was still captured by DCs via CLR-dependent mechanisms before migrating out of the tissue [113]. Hence, while DC (and T-cell) infection may be a major driving force in establishing infection, this emphasizes the complexity and diversity of mechanisms HIV uses during transmission. Thus, we face a dire need to identify strategies that can block the multitude of receptors and post-attachment/fusion processes that can be utilized by HIV as it interacts with different cell types to ensure that all pathways are impeded.

Taking this work *in vivo*, several macaque studies demonstrated that the targeting of HIV envelope by neutralizing anti-envelope antibodies and glycosylation-specific compounds, like the b12 monoclonal antibody and CNV (respectively), could block immunodeficiency virus infection across the mucosal surfaces [1–4, 196–200]. This is in agreement with *in vitro* studies demonstrating the ability of anti-envelope antibodies and CNV to block infection of DCs and amplification in the DC–T-cell milieu (above). More recently, compounds that target the CCRs needed for productive infection were also found to impede vaginal infection in macaques [108, 201, 202]. It is critical to note that the amounts of the blocking agents used in these studies are significant and probably not feasible for microbicide development *per se*, but provide proof of the principle that infection can be prevented through such specific approaches.

Another alternative is to use agents that act broadly against all of the mechanisms used for capture and infection of DCs and T cells (and other leukocytes), such as the sulfated polysaccharides [10, 11]. Carrageenan successfully prevents HSV-2 infection in mice [203, 204], blocks capture and entry of virus into DCs (MP unpublished observations), and exhibits ~70% efficacy in preventing vaginal infection of macaques with SIV (David Phillips, personal communication). In a similar manner, PRO-2000, another sulfated polymer, prevented HIV infection of skin-derived DC–T-cell mixtures (MP, unpublished observations) and was reported to protect 50–75% of macaques following vaginal challenge with $SHIV_{89.6PD}$ [205]. Another more general-acting strategy that putatively acts by creating a protective film over the epithelia, cellulose acetate phthalate (CAP), also significantly limited vaginal infection of macaques after repeated low-dose exposures to SHIV162P3 [206]. Broad-acting strategies like these will probably prove effective against immunodeficiency viruses as well as other pathogens to further limit HIV spread.

Given that HIV replication occurs within the local mucosal tissues, whether in DCs or $CD4^+$ T cells (macrophages), and appears to precede systemic dissemination of virus in certain circumstances, microbicides and/or preventative measures that restrict viral replication locally, beyond initial virus attachment/fusion, may be another strategy to curb HIV (Fig. 38.4). Work by Van Rompay and colleagues [207] highlighted this by topically orally applying tenofovir to protect subsequent oral challenge using SIV in newborn macaques.

38.6.2
Using DCs to Boost Immunity

Broad-acting systemic and mucosal T-cell and B-cell responses will be needed to prevent new, and control existing, infections (Fig. 38.4). As detailed in other Chapters within this book, identifying ways to properly target a vaccine to activated DCs represents one of the most promising ways to improve vaccine efficacy [208], especially against HIV [209]. To achieve this, researchers are exploring several avenues, including the use of viral vectors to directly target the DCs, chemoattractants to recruit more DCs to the site of vaccination, and adjuvants to activate them.

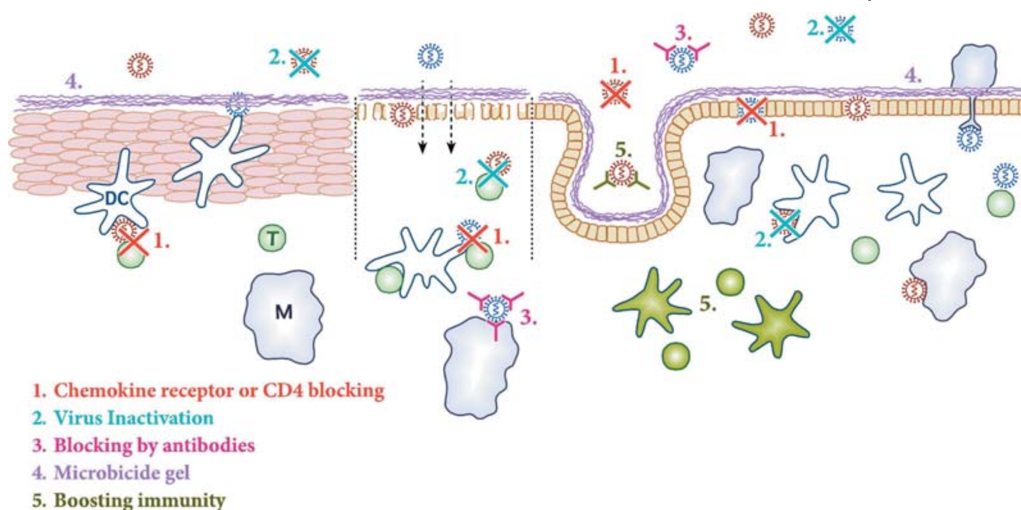


Fig. 38.4 Antiviral actions at the surfaces and in the tissues. Multiple strategies are being explored to block the virus entry and spread. One approach consists of blocking infection using CD4 or CCR antagonists, which bind to the permissive cells. Preventing binding of envelope to host cells can be achieved using neutralizing anti-envelope antibodies or agents like CNV. Another type of microbicide would prevent virus entry by nonspecific saturation of the virus-cell interactions (e.g. sulfated polymers). Inclusion of antiviral drugs that prevent infection by targeting infectious virus would improve these strategies that preferentially block the initial virus attachment. Boosting the host immunity by vaccination will lead to the emergence (and/or expansion) of HIV-specific immune cells at the mucosal sites that can directly target the virus (e.g. antibodies) and virus-infected cells (e.g. cytotoxic T cells). Boosted innate immune responses could also limit incoming infections as well as reduce the spread of infectious virus being shed.

38.6.2.1 DC-induced Primary Responses for Preventative HIV Vaccines

Support for the idea that access to activated DCs can be a rate-limiting aspect of an HIV vaccine's efficacy came from a recent study demonstrating that intramuscular administration of plasmids encoding MIP-1α/CCL3 and Flt3L successfully recruited, expanded, and activated DCs at the site of inoculation and coincidently boosted the responses against a co-administered DNA vaccine [210]. Moreover, these mice exhibited enhanced resistance to infection with recombinant HIV envelope-expressing *Vaccinia* virus. Importantly, the type of DNA vaccine may also influence the quality of the response induced, since HIV gp120 DNA was shown to induce IL-10-driven Th2 responses (avoiding DC maturation) while an influenza hemagglutinin DNA antigen induced an IL-12-dominated Th1 response [211]. In a separate murine study, re-injection of human DCs directly loaded with HSV-1 amplicons expressing HIV gp120 into hu-PBL-NOD/SCID mice was shown to induce cellular and humoral immunity, partially protecting against infectious HIV challenge [212].

Increasing research is now being performed using the SIV-macaque system. Immature macaque moDCs, transduced with SIV gag-recombinant adenovirus vectors and matured with CD40L, were re-injected intradermally or intranodally into the donor animals and were shown to induce SIV-specific T-cell responses [213]. Using SIV-recombinant canarypox vectors, we similarly demonstrated the induction of primary SIV-specific IFN-γ-producing cells by *ex vivo*-loaded moDCs and that more robust responses were observed when mature DCs or additional helper antigens were used [214]. Notably, no SIV-specific antibody responses were detected in the plasma of the animals immunized with recombinant canarypox-infected DCs (even after repeated immunizations). Knowing that moDCs bearing inactivated virions can stimulate $CD4^+$ and $CD8^+$ SIV-specific T cells [39], we subsequently found that subcutaneously re-injected moDCs loaded with AT-2 SIV can elicit SIV-specific IFN-γ-secreting cells in naïve animals (MP, unpublished observations). Together, these studies provide proof of the principle that targeting an immunodeficiency virus antigen to DCs will empower the DCs to elicit antiviral T- and B-cell responses.

Improved strategies to directly access circulating DCs *in vivo* will, however, be needed to truly advance viable vaccines. As noted earlier, PDC and MDC biology in the macaque is comparable to that in humans and macaque DCs are responsive to agents that mobilize and/or activate DCs [33, 35, 36, 64]. Thus, vaccine strategies employing adjuvants to recruit and activate DCs at immunization sites will probably improve vaccine effectiveness. Topically applied DNA vaccines probably capitalize on some of the circulating DC biology thereby resulting in encouraging virus-specific immune responses [215]. Circulating DC function and the SIV-specific responses they stimulate can be enhanced through CpG ISS-ODN-mediated activation [36]. CpG-mediated effects were also associated with innate and adaptive antiviral responses detected in one DNA vaccine study [216], further underscoring the potential importance of identifying how to properly harness the DC system to trigger innate and adaptive responses.

38.6.2.2 **DC-based Therapeutic Control of Existing Immunodeficiency Virus Infection**

Another rationale is that the virus-specific immunity, induced as a result of infection, needs to be boosted to help control existing infections and that DC-based approaches represent a promising avenue through which this could be achieved [217]. The first evidence for this possibility was demonstrated by Lu et al. when they reported that AT-2 SIV-loaded moDCs enhanced SIV-specific immune responses in infected macaques, concurrent with reduced viral loads [218]. This was subsequently supported by another group's observations [219]. Lu et al. also reported that moDCs loaded with autologous, AT-2-inactivated HIV augmented the HIV-specific immunity while lowering plasma viral loads in these individuals upon re-injection [220]. CpG ISS-ODNs also represent a potentially potent modality via which innate and adaptive DC activities can be enhanced to increase antiviral immunity in HIV-infected people [221]. This concept is further supported by our data

that DCs and B cells in healthy, infected macaques exhibit normal responsiveness to CpG ISS-ODN stimulation *in vitro* and *in vivo* [36, 65]. These data provide promise for the utilization of DCs in the development of therapeutic vaccines.

38.7 Summary and Future Perspectives

The consequence of DC-immunodeficiency virus communication is a paradoxical event. The same cells that should capture the virus and present viral determinants to the immune system to mount robust antiviral responses in fact elicit substandard immunity while promoting virus amplification and dissemination. Research needs to continue to fully understand the intricacies of DC–virus interplay to enable the development of effective, targeted approaches that will tip the balance of this irony. We need to empower DCs to stimulate strong antiviral T- and B-cell responses as well as effective innate responses, coincident with limiting DC-driven infection. Together, this will assist in the advancement of preventative vaccine and microbicide approaches in addition to progressing DC-based immune therapies to help control virus replication in, and spread from, infected persons.

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39
Cytomegalovirus Infection of Dendritic Cells

Brigitte Sénéchal and James W. Young

39.1
Introduction

Human cytomegalovirus (HCMV) is a major opportunistic pathogen and an important cause of birth defects. HCMV is an endemic β-herpesvirus that comprises a large double stranded DNA molecule containing more than 200 open reading frames. HCMV infects 50 to 90% of the population, usually with mild or asymptomatic primary infections. It establishes a life-long infection, however, despite strong immunity developed by the host. This immunity controls viral replication, although intermittent shedding does occur. When HCMV reactivates and replicates under conditions of immune suppression, e.g., hematopoietic stem cell or solid organ grafting or during infection with HIV, it can result in substantial morbidity and mortality despite treatment with effective antiviral drugs [1]. Thus while CMV-specific immunity developed by the healthy host limits CMV infection, it cannot clear the virus or prevent the viral shedding that ensures spread within the population. Like other herpesviruses, HCMV has therefore adapted to its host and has evolved multiple strategies to escape innate and adaptive immunity [2].

39.2
HCMV Induces Immunosuppression

Acute HCMV infection exerts transient T-cellular immunosuppression in healthy individuals. Although seroconversion occurs rapidly after primary infection, low specific cellular responsiveness against HCMV and other pathogens has been observed during the acute phase of HCMV infection, in contrast to healthy CMV-seropositive persons [3]. Cellular immune suppression induced by CMV can sometimes persist for more than one year, concomitant with CMV DNA detection in the blood and shedding in urine and saliva [3]. Recently, this cellular immune defect has been defined as a lack of IFN-γ secreting CD4+ T lymphocytes in healthy young children [4], whereas CMV-specific CD8+ T lymphocytes are unaffected [5].

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Acute HCMV infection can also occur in immunosuppressed patients as a result of HCMV reactivation, primary infection, or reinfection. HCMV infection and/or positive serology (recipient and/or donor) are independent risk factors for developing serious secondary bacterial or fungal infections after transplantation [6, 7]. Thus HCMV infection increases immune suppression in transplanted patients.

39.3
A Role for Dendritic Cells in the Pathology of CMV Infection

Numerous viruses target DCs. Some viral infections induce DC maturation and stimulate the development of virus-specific T-cell responses [8, 9]. Other viral infections result in immune suppression by lysis of DCs, impairment of DC function without lysis, or release of infectious virus to bystander cells [10–13].

While T-cells are a key component of the antiviral immune response and T-cell lymphopenia is an obvious risk factor for HCMV disease, DCs are critical initiators of cellular immunity against viral pathogens like HCMV. The sequelae of HCMV infection of DCs could also account for much of the additional immune suppression associated with reactivation in immunocompromised patients or with acute infection of healthy individuals.

39.4
The Myeloid Lineage and Monocytes are Major Sites of HCMV Latency

Hematopoietic progenitors, including those of the granulocyte–macrophage lineage, can exhibit latent infection *in vitro* and *in vivo* but fail to support HCMV replication [14, 15]. The infection is not entirely silent [16], however, as latency-associated transcripts have been detected in such hematopoietic progenitors [17]. Latency-associated proteins may play a role in escaping immune surveillance by decreasing cell surface class II MHC expression or by expressing an IL-10 homolog [18, 19].

Monocytes are the main cells infected in the peripheral blood, but these cells fail to support HCMV replication [20, 21] Monocytes can differentiate *in vitro* into fully permissive macrophages, however, which can produce new viral particles confined to cytoplasmic vesicles [22–24]. Monocytes can also differentiate into permissive dendritic cells [25–27] and CD34+ progenitors can differentiate into permissive Langherans cells [28].

Endogenous HCMV can be recovered from healthy seropositive donors after an allogeneic stimulation *in vitro* of cells bearing macrophage and dendritic cell markers [29]. Other stimuli, like concanavalin-A or IFN-γ, may induce immediate early phase replication but not a complete viral cycle [30, 31]. Mononuclear cells isolated from patients highly infected by HCMV and differentiated into mature DCs maintain cell-associated virus but do not release infectious particles into the supernatant

during culture *in vitro* [32]. Tissue macrophages from infected patient also express viral proteins representing all stages of permissive HCMV infection, indicating that these cells support the complete viral replication cycle [33].

39.5
Human Dendritic Cells are a Potential Target for HCMV

Conventional or myeloid DCs include constitutively circulating blood DCs, as well as resident populations of Langerhans cells (LCs) and dermal-interstitial DCs (DDC-IDCs). The latter two can be generated *in vitro* from CD34+ hematopoietic progenitors using exogenous cytokine supplements, just as blood monocytes can differentiate into monocyte-derived DCs (moDCs). The stage of myeloid differentiation determines permissiveness for HCMV infection. Bone marrow progenitors serve as reservoirs for HCMV without lytic gene expression and transmit the viral genome to monocytes. Monocytes circulate only briefly and may reactivate latent CMV upon leaving the circulation and undergoing tissue-specific differentiation into macrophages or dendritic cells (Fig. 39.1).

HCMV can infect a broad variety of cell types including endothelial cells, epithelial cells, fibroblasts, hematopoietic cells and smooth muscle cells. Indeed, HCMV is capable of causing disease in nearly every organ system in immunocompromised patients. This broad pathogenic tropism has made receptor identification extremely difficult; and multiple candidates may participate in HCMV attachment and entry like heparan sulfate, annexin II, the epidermal growth factor receptor, and integrins [34–37].

DCs express a C-type lectin receptor called DC-SIGN, however, that binds the carbohydrate moieties of glycoprotein self antigens and pathogens for processing and presentation on MHC molecules. Sampling and presentation of self and harmless environmental antigens maintain peripheral tolerance in the steady state

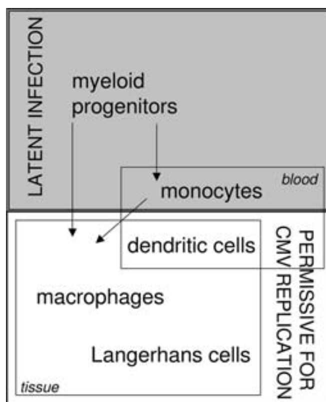

Fig. 39.1 The myeloid lineage is an important reservoir of CMV.

[38, 39]. Some pathogens also exploit these receptors to evade immune activation, e.g., HIV and DC-SIGN [40–42]. DC-SIGN can also bind the HCMV envelope gpB with a high affinity and contribute to HCMV attachment and entry into DCs at low rates of infection [43]. Disruption of the steady state by simultaneous exposure to an activating stimulus like TLR-binding ligands or CD40L can override any tolerizing function of C-type lectin receptors and lead to immune activation [44, 45].

39.6
In vitro Evidence for HCMV Entry and Replication into Dendritic Cells

Studying HCMV susceptibility *in vitro* is challenging because there are a number of HCMV strains available that can lead to different results. HCMV strains fall into two broad categories, however. Laboratory strains, like AD169, Towne, and other fibroblast-adapted strains have been frequently passaged on human fibroblasts. Some strains have deleted regions in their genome and display a narrow cell tropism. In contrast, HCMV isolates passaged on endothelial cells can infect a broader variety of cell types [46, 47].

Monocyte-derived DCs are permissive to HCMV infection (Fig. 39.2). Immature DCs are fully permissive to HCMV infection when E-strains are used [25-27, 32]. Cultures of Infected immature moDCs display a typical cytopathic and cytomegalic effect with enlarged, refractile cells. Infection yields detectable Immediate early (IE), early (E), and late (L) viral proteins, confirming each replication step and an increase in viral load over time. The viral cycle is completed by the production of new infectious viral particles, which leads to DC lysis and death. In contrast, fibroblast-adapted strains cannot replicate efficiently in immature DCs *in vitro*, and so very few if any infected DCs can be detected.

Susceptibility of mature moDCs has also been examined. Endothelial cell-passaged strains infect mature DCs with less efficiency than immature DCs, at the same multiplicity of infection (MOI). Whereas infection rates in immature DCs can reach 80%, they barely attain 50% in mature DCs. Such disparities in rates of infection become even more pronounced at lower MOIs. IE, E, and L steps of replication are again achieved together with an increase in viral load [26, 27, 32]. Infected mature DCs do not undergo cytomegalic changes and are not lysed by viral release [26, 32]. Hence, infectious virus cannot be recovered from culture supernatants but can be recovered by sonication of infected mature DCs [26, 32]. Akin to macrophages, virions might be confined to cytoplasmic vesicles [24]. Little data exist regarding infection of mature DCs with fibroblast-adapted HCMV strains, which can gain entry into DCs but do not replicate efficiently [26, 27].

DCs, or at least moDCs, are therefore highly susceptible to HCMV infection. Lysis of immature moDCs by productive HCMV infection contributes to viral dissemination and immune suppression. Infection and replication of HCMV in mature moDCs, which in contrast is nonlytic and nonproductive, can thus establish a viral reservoir in these important antigen-presenting cells.

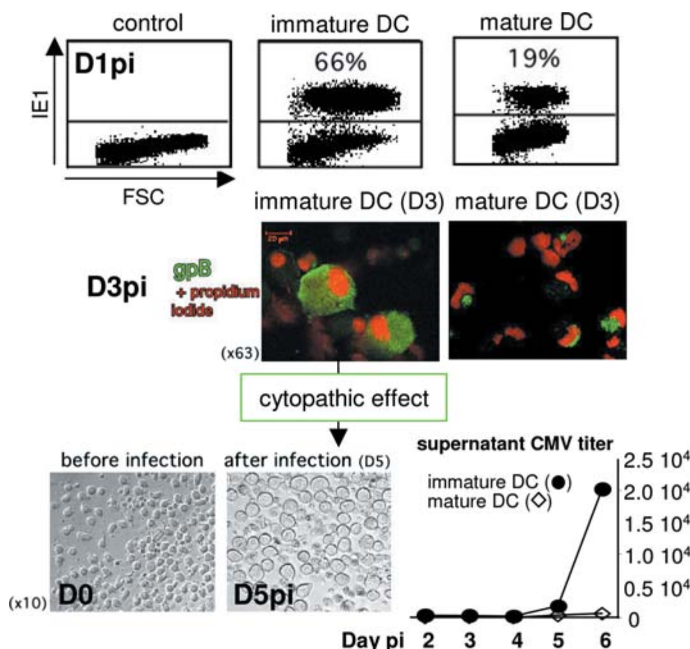

Fig. 39.2 DCs are permissive for CMV infection. Immature and mature monocyte-derived DCs (moDCs) express immediate-early-1 (IE1) antigen at day 1 post infection (D1pi). IE1 expression is always greater in immature than mature moDCs, however, at the same multiplicity of infection (top panel). A late replication step is also achieved as proven by the expression of glycoprotein B (FITC) with propidium iodide for nuclear staining (middle panel), with different patterns between immature and mature moDCs. Indeed immature moDCs, but not mature moDCs, display the typical cytopathic effect (D5pi versus D0 in the photomicrographs, bottom panel) and release infectious viral particles during late replication steps (HCMV supernatant titer, far right, bottom panel). (These data were originally published in Senechal et al., Infection of mature monocyte-derived dendritic cells with human cytomegalovirus inhibits stimulation of T-cell proliferation via the release of soluble CD83. Blood. **2004**, 103: 4207-4215. © 2004 The American Society of Hematology.)

39.7
HCMV Impairs the Function of Immature Dendritic Cells

Very soon after infection of immature DCs, HCMV induces a viral replication dependent downregulation of class I and class II MHC, CD80, CD86, and CD40 expression [26, 27, 48]. The diminution of these epitopes increases over time and is selective, rather than mediated by a broader shutdown of protein synthesis. Bystander HCMV-negative DCs express the same or higher levels of these molecules compared with mock-infected cells, indicating apparent activation by contact or association with infected DCs in the same HCMV-exposed cultures [26, 48].

HCMV-infected immature DCs are resistant to maturation stimuli, which can neither upregulate MHC and co-stimulatory molecule expression, nor induce IL-

12, TNF-α, IL-10, or IL-6 secretion [26, 27, 48]. Interestingly, maturation stimuli upregulate MHC and co-stimulatory molecules expression by uninfected DCs from the same HCMV-exposed cultures, but cannot induce secretion of inflammatory cytokines. A putative explanation is that a soluble factor suppresses cytokine secretion of all cells in culture, whereas HCMV replication decreases expression of DC surface markers and therefore affects only infected cells.

Functional assays confirm that HCMV infection decreases immunostimulatory capacity of immature DCs. HCMV infection of immature DCs impairs their ability to induce class I MHC-mediated T-cell responses to recall antigens like Influenza virus [48]. HCMV infection also decreases their ability to stimulate alloreactive T-cells. Decreased capacity to stimulate T-cells occurs at low rates of infection [26], consistent with the role of a soluble factor in impairing function of bystander non-infected DCs [27].

HCMV infection also alters the normal pattern of DC migration. HCMV-infected immature DCs downregulate the inflammatory CC chemokines, CCR1 and CCR5, and fail to upregulate CCR7 with activation. Infected DCs consequently do not migrate toward the chemoattractants CCL3, CCL5, CCL19, and CCL21 [49, 50]. HCMV-negative bystander DCs can migrate, however, in response to CCL19 and CCL21 [49]. Indeed, bystander uninfected DCs upregulate CCR7 when stimulated with LPS or HCMV infected fibroblasts and may stimulate lymph node T-cells by crosspresentation [49].

HCMV infection of immature DCs therefore induces immune suppression through multiple mechanisms. HCMV-infected DC express low levels of MHC, co-stimulatory, and CD40 molecules and resist maturation stimuli in a replication dependent manner. They display a decreased capacity to stimulate T-cells and most likely have impaired migration to draining lymph nodes. Immature DCs allow viral replication to proceed with exposure of neighboring cells to new infectious virus. Infected DCs also deliver a negative signal to bystander uninfected DCs with a mixed but altered effect on normal maturation.

39.8 HCMV Impairs the Function of Mature Dendritic Cells

The effect of HCMV-infection is less disruptive for mature than immature DCs. Expression of MHC and co-stimulatory molecules is slightly lower on HCMV positive cells than on bystander uninfected cells [26], and CCR7 is not downregulated by infection [27, 50]. HCMV does, however, exert a dramatic effect on the expression of CD83, which disappears from the cell surface within 24 hours after infection [26].

HCMV infection of mature DCs inhibits their capacity to stimulate alloreactive T-cells by mechanisms other than changes in the maturation and activation phenotype [26, 27, 32]. Deletional and non-deletional mechanisms have been proposed. HCMV induces expression of CD95-L (Fas-L) and TRAIL (TNF-related apoptosis-

induced ligands) on the surface of mature DCs, which could in theory kill activated T-cells, thus constituting a deletional mechanism [32]. Most of the T-cells survive, however, and cannot respond to a second stimulation in the absence of virus. Furthermore, neither caspase-8 deficient T-cells nor blockade of Fas/Fas-L interactions restore T-cell proliferation [26, 32]. Thus a soluble inhibitory factor has been strongly suggested to account for inhibition of mature DC stimulatory capacity, even at low rates of infection. Indeed, virus-free supernatant from infected mature DC culture can inhibit alloreactive T-cell proliferation in a dose dependent manner [26, 27]. Efforts to identify a soluble inhibitory factor have excluded IL-10 receptor stimulation (hIL-10 or vIL-10) and TGF-β secretion by infected DCs [26].

The extracellular domain of CD83 expressed by mature DCs is soluble and can inhibits DC immunostimulatory capacity upon released by activated DCs [51, 52]. CD83 expression rapidly decreases on the surface of HCMV-infected mature DCs, but normal levels are still expressed in the cytoplasm. At the same time the supernatants of infected cultures contain high amounts of soluble CD83, compared with supernatants from mock infected culture. Immunodepletion of soluble CD83 from infected culture supernatant, but not from uninfected cultures, restores normal alloreactive T-cell proliferation [26]. Thus release of soluble CD83 by infected mature DCs may inhibit the immunostimulatory capacity of both infected and non-infected mature DCs. Interestingly soluble CD83-inducing immunosuppression might be a mechanism shared with other herpesviruses. VZV and HSV-1 infect and impair the function of mature DCs, which display low CD83 expression, although CD83 is degraded in HSV-1 infected cells [10, 53].

Although HCMV-infected immature DCs have lost normal capacity to mature, migrate, and stimulate T-cells, they replicate HCMV and may in turn infect mature DCs. Infected mature DCs replicate the virus without lysis and may act as a stable reservoir of infectious virions. Furthermore, HCMV-infected mature DCs have a diminished capacity to stimulate T-cells, in large part because they secrete soluble factors, including CD83, that inhibit host immunity. The migration potential of HCMV-infected mature DCs remains to be examined, although they express CCR7.

39.9
Langerhans-type Dendritic Cells are also Permissive to HCMV

Langerhans cells (LCs) are DCs that reside in epithelial tissues. LCs can be generated *in vitro* from CD34+ hematopoietic progenitor cells, under the aegis of c-*kit*-ligand or stem cell factor, Flt3-ligand, GM-CSF, TNF-α, and TGF-β [54–57]. In contrast to moDCs, immature LCs are poorly susceptible to infection *in vitro* by endothelial cell-adapted HCMV strains. In further contrast to moDCs, susceptibility increases greatly after maturation by CD40L stimulation [28]. Mature LCs are fully permissive to HCMV infection, which induces a slight downregulation of class II MHC and co-stimulatory molecules. HCMV induces a much greater downregula-

tion of class I MHC and an even more dramatic alteration of surface CD83 expression, similar to what we observed on mature monocyte-derived DCs. Accordingly, HCMV-infected Langerhans cells also display a reduced capacity to stimulate alloreactive T-cells [28], possibly through a similar soluble CD83-mediated suppression.

39.10
Importance of Viral IL-10 in HCMV-induced Immunosuppression

HCMV exploits the IL-10 signaling pathway by expressing a functional viral IL-10 homolog (CMV-IL-10), which shares only 27% sequence identity with huIL-10 yet signals through IL-10R1 and IL-10R2 [58-61]. The UL111a gene encodes CMV-IL-10 and alternative splicing generates another huIL-10 homolog in latently infected cells. This would support another means of immune escape by latent virus [19].

Recombinant CMV-IL-10 inhibits maturation of immature DCs in response to LPS, blocking the upregulation of MHC and co-stimulatory molecules and secretion of IL-12, IL-6, and TNF-α [60, 62]. DCs treated with LPS in the presence of CMV-IL10 maintain active endocytosis characteristic of immature DCs and have a diminished potency for stimulating T-cells compared with mature DCs [60]. CMV-IL-10-treated DCs have also been shown to upregulate indoleamine 2,3-dioxygenase (IDO), a proposed tolerogenic enzyme that could further support HCMV-induced immune suppression [60]. CMV-IL10 also inhibits LPS-induced upregulation of the anti-apoptotic factors $cFLIP_L$ and $Bcl\text{-}X_L$ [60]. HCMV inhibition of DC maturation can therefore be a direct effect of viral replication and lysis of infected, immature DCs. There is also an indirect effect on non-infected DCs that impairs their survival and is mediated by CMV-IL-10.

39.11
CMV Infection of Dendritic Cells in the Mouse Model

CMV infection is species-specific, and there are no experimental animal models for the study of HCMV pathogenesis. Because mouse cytomegalovirus (MCMV) and HCMV share similarities in structure and biology, however, MCMV infection can approximate at least some aspects of CMV pathogenesis in humans.

The mouse model has yielded more information about control of CMV by innate immunity than have studies *in vitro* with human cells. Mouse strain susceptibility to MCMV is in fact determined by genetic factors regulating natural killer (NK) cell activity, which is crucial to the early control of MCMV infection. Two independent genomic domains on chromosome 6, *Cmv1* and *Ly49H*, determine susceptibility or resistance to MCMV infection in some mouse strains [63–66], while the antiviral activity of NK cells can be independent of these allelic loci in other strains [67].

MCMV is capable of subverting the immune system by multiple mechanisms, and monocytes and macrophages are important for its dissemination and pathog-

enicity. Immature and mature mouse DCs are susceptible to MCMV *in vitro* [68, 69]. Viral replication occurs with production of infectious virus, although cell viability is untouched [68]. Further study has shown that MCMV prevents induction of DC apoptosis in association with upregulation of antiapoptotic Bcl-2 family proteins [70]. Models *in vivo* and *in vitro* have shown that splenic DCs (CD11c^{high} CD11b+ and CD11c^{high} CD8α+) and plasmacytoid DCs (PDCs) are activated early after MCMV infection [68, 69, 71], but this changes at later time points after further viral replication in DCs [68].

During acute MCMV infection *in vivo*, PDCs are the major producers of type I IFNs and IL-12. PDCs limit IL-12 secretion by CD11c^{high} CD11b+ DCs through production of type-I IFNs [72], whereas CD11c^{high} CD11b+ DCs can secrete high levels of IL-12 in mice lacking PDCs or the type-I IFN pathway [73]. MCMV also induces secretion of TNF-α and MIP-1-α by PDCs [71]. High levels of type-I IFNs induce maturation of all DC populations, promote accumulation of PDCs, and amplify innate immune responses that limit viral infection. Most importantly, only PDCs but not CD11c^{high} splenic DCs are able to activate NK cells, which in turn control viral replication [71]. Stimulated Ly49H+ NK cells are later needed to maintain CD11c^{high} DCs, however, which otherwise disappear from the spleen of susceptible animals six days after infection [68]. Conversely, CD11c^{high} CD8α+ DCs are later required for Ly49H+ NK cell expansion mediated by IL-12 and IL-18 [74].

MCMV activates PDCs and splenic DCs through TLR9 [75]. Absence of TLR9/MyD88 signaling reduces the levels of IFN-α, IL-12, and MIP-1-α in the serum and alters early NK cell activation [75, 76]. Hence MyD88$^{-/-}$ and TLR9$^{-/-}$ mice control MCMV replication inefficiently *in vivo* and die rapidly [75, 76]. MCMV also activates TLR3, which signals through the Trif pathway. Absence of TLR3 activation during MCMV infection has a less dramatic effect on cytokine secretion, viral load, and survival compared with wild type mice, than does disruption of the TLR9/MyD88 pathway [76].

At early time points after infection *in vivo*, MCMV-induced maturation licenses splenic DCs to stimulate naive CD8 T-cells *in vitro* [71]. Thorough study of infected CD11c^{high} DCs *in vitro* shows that MCMV replication impairs DC functions at later time points after infection [68]. Indeed, infected DCs first display an activated phenotype, after which MCMV infection decreases expression of class I and II MHC, CD80, CD86, and CD40. A viral factor responsible for the specific decrease of CD86 expression has been recently identified [77]. Similar to the human situation, maturation stimuli fail to increase the expression of MHC- or co-stimulatory molecules, or induce high levels of IL-12 or IL-2 secretion [68]. Infected DCs display a reduced capacity for antigen uptake and a low capacity to stimulate alloreactive T-cells [68]. After infection *in vivo* with recombinant MCMV strains, investigators have observed that MCMV infects variable proportions of splenic DCs (<1% up to 20%) and preferentially targets CD11c^{high} CD8α+ DCs [68, 71]. Whether infected CD11c^{high} CD8α+ DCs interfere *in vivo* with T-cell priming remains to be determined.

39.12 Conclusion

HCMV targets DCs and thereby promotes viral replication and spread, establishes a stable viral reservoir, and suppresses immune responses by a variety of mechanisms (Fig. 39.3). HCMV infection clearly impairs DC functions by direct, replica-

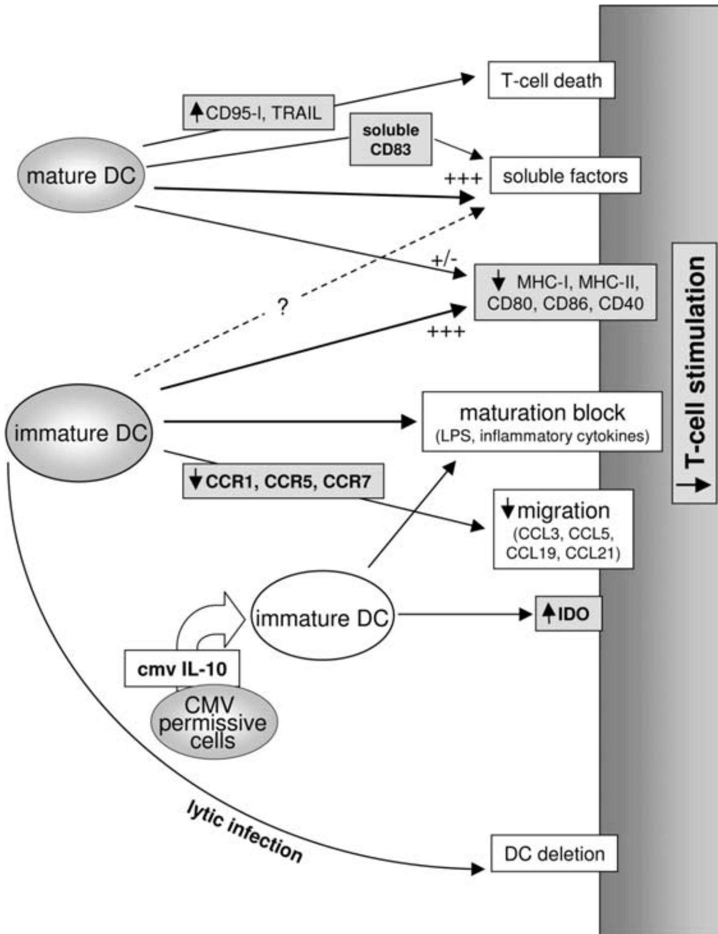

Fig. 39.3 Schematic summary of the multiple mechanisms used by CMV to dampen DC function and T cell-mediated immune responses. CMV-infected cells are represented by shaded circles and bystander non-infected cells by open circles. Immunosuppression induced by CMV can be either a direct effect of its replication in DCs or an indirect effect on bystander uninfected cells due to the release of soluble inhibitory factors. Shaded text boxes indicate mechanisms, whereas open text boxes indicate molecules that are up- or down-regulated as a result of HCMV infection. Although plasmacytoid dendritic cells and NK cells are important to limit infection early on (based on the mouse model), development of CTL is important for resolution of acute infections and to maintain viral latency over a long period of time.

tion-dependent means, as well as by indirect mechanisms involving soluble factors like CD83 and CMV-IL-10, Bystander immune suppression of non-infected DCs *in vivo* may delay development of a strong CMV-specific T-cell response, while viral load is increasing and establishing sites of latent infection. Both mechanisms may therefore help establish life-long HCMV infection. Furthermore, impairment of bystander DCs would also promote secondary infection in immunosuppressed patients with an acute HCMV infection. Latently infected myeloid progenitors and monocytes may reactivate endogenous HCMV upon strong inflammatory stimulation. HCMV-specific soluble and cellular immunity is well defined and keeps HCMV infection at a subclinical level in the healthy host. Whether HCMV infection of DCs interferes *in vivo* with the priming of HCMV-specific T-cells is not known. Investigators have suggested that DCs could overcome direct viral suppression of DC-mediated immunity by cross-presenting viral antigens [78, 79]. Cross-presentation might be influenced by soluble inhibitory factors released during HCMV infection, however [80]. Data on innate immunity against CMV in humans are scarce, but the mouse model has provided important information regarding the crucial role of innate immunity against MCMV at the earliest time points after infection.

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40
Interactions of Hemorrhagic Fever Viruses with Dendritic Cells

Stefan Pöhlmann

40.1
Introduction

Several RNA viruses cause disease with hemorrhagic manifestations in humans. Hemorrhagic fever viruses (HFV) are found in the families: filoviridae, arenaviridae, bunyaviridae and flaviviridae, which comprise enveloped viruses with a RNA genome (reviewed in [1]). Vaccines or effective treatment against most of these viruses are not available. Filoviruses are probably the most notorious HFVs. They are endemic in Africa and induce severe disease in infected humans. The natural host for filoviruses is unknown (reviewed in [2, 3]). The first publicly noted filovirus outbreak occurred in primate centers in Yugoslavia and Germany in 1967 and to date 1500 deaths due to filovirus infection have been registered. Other HFVs like dengue virus (DEN) receive less public attention, however, the threat to public health posed by DEN is more imminent. Thus, the geographic distribution of DEN has broadened and there has been a massive increase in the number of infections during the last five decades (reviewed in [4–6]), suggesting that the virus might evolve into a challenge for health systems around the globe. HFVs are also potential agents of bioterrorism and several have been weaponized (reviewed in [1]), posing an unpredictable risk to human health.

Dendritic cells (DCs) are the principle antigen presenting cells and are uniquely capable of stimulating naïve T cells, thereby initiating adaptive immune responses. Immature DCs line all body surfaces, which constantly come into contact with pathogens, and are particularly adept in recognizing and taking up antigen (reviewed in [7, 8]). Activated DCs leave the periphery and migrate into lymphoid tissue. During the migration the DCs mature – they downregulate their antigen uptake machinery and upregulate the expression of molecules required for antigen presentation. Within lymphoid tissue DCs present antigen via MHC to T cells (reviewed in [7–9]), in a process that involves a variety of cell–cell adhesion and costimulatory molecules. Thus, DCs are sentinels of the immune system and provide a powerful defense against invading pathogens.

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Several viruses specifically target DCs and interfere with DC function to promote their spread in and between hosts (reviewed in [10]). Here, recent studies on filovirus, DEN, Lassa virus (LV) and *Hantavirus* (HTV) interactions with DCs are discussed and an introduction to the molecular biology, epidemiology and pathology of these viruses is presented. Filoviruses and DEN are discussed in more detail, since these viruses seem to have evolved a common mechanism for targeting DCs: they engage the lectin DC-SIGN, a universal attachment factor used by several viral and nonviral pathogens for entry into DCs.

40.2 Filoviruses

40.2.1 Pathology and Epidemiology

Filovirus infection produces a severe form of hemorrhagic fever in humans. Hallmarks of the disease are fluid distribution problems, hypotension, altered coagulation and hemorrhages (reviewed in [11–17]). However, blood loss due to the latter complication is not sufficient to account for a fatal outcome of the disease. Early symptoms develop between a few days and up to three weeks after infection and include fever, chills, myalgia and malaise (reviewed in [11–17]). As the disease progresses a multitude of clinical manifestations is observed, including rash, vomiting, abdominal pain and coma. At the end stage of the disease, patients develop a condition similar to septic shock. Most affected individuals succumb to the infection about a week after the onset of clinical symptoms. No vaccines or treatments against filovirus infection are currently approved for use in humans.

The family filoviridae contains two genera, *Ebolavirus* (EBOV) and *Marburgvirus* (MARV) (Table 40.1). Lake Victoria MARV is the only subspecies of the MARV genus, while four subspecies of EBOV have been described, and termed, according to the place of their emergence, EBOV Zaire (ZEBOV), Sudan (SEBOV), Ivory Coast (ICEBOV) and Reston (REBOV). The EBOV subspecies exhibit differential pathogenicity (reviewed in [2]) and the determinants of this variable pathogenicity have not been fully elucidated. Several outbreaks of EBOV and MARV have occurred in

Tab. 40.1 HFVs discussed in the present review.

Family	*Genus*	*Envelope*	*Genome*	*Vector*	*Disease*
Filoviridae	EBOV, MARV	+	RNA, –	?	EHF, MHF
Arenaviridae	LV	+	RNA, +/–*	Rodent	LF
Flaviridae	DEN	+	RNA, +	Mosquito	DHF, DF
Bunyaviridae	HTV	+	RNA, –**	Rodent	HFRS, HPS

* 2 segments
** 3 segments

Africa and it is believed that these viruses replicate in animal reservoirs from which they are occasionally transmitted to humans (reviewed in [2, 3]). Despite several interesting leads, including bats and plants, such natural reservoirs remain to be identified (reviewed in [2, 3]). Note that EBOV is endemic in great apes in Africa and is responsible for a steep decline in the animal population [18].

40.2.2
Replication

Filoviruses are filamentous, enveloped RNA viruses that contain a single, genomic RNA molecule of negative polarity (Table 40.1). The *Ebolavirus* genome harbors seven open reading frames (ORFs) for viral proteins and is organized as follows: 3′ leader, nucleoprotein (NP), virion protein (VP) 35 (VP35), VP40, glycoprotein (GP), VP30, VP24, polymerase protein L and 5' trailer (reviewed in [19, 20]). All viral proteins, expect for a soluble form of GP (sGP, see below) are packaged into progeny virions. NP, VP30, VP35 and L are associated with the viral RNA and play a role in replication of genomic RNA and production of messenger RNAs encoding viral proteins, while GP, VP40 and VP24 associate with the viral membrane and facilitate viral entry and release.

Filovirus infection of susceptible cells is initialized by interactions of the vial GP with cellular membrane proteins, which ultimately results in fusion of the viral and a cellular membrane. Albeit several candidate molecules have been proposed [21, 22], the cellular receptor(s) for filoviruses remain(s) elusive [23]. The heavily glycosylated GP is synthesized as a precursor protein (GP_0), which is cleaved by cellular furin like proteases into the covalently linked subunits GP_1 and GP_2 reviewed in [24, 25]. GP_1 mediates interactions with cellular attachment factors (described in more detail below) and receptors, while GP_2 harbors the functional elements required for membrane fusion. GP_1 and GP_2 form heterodimers, which are linked by disulfide bonds, and GP_1–GP_2 dimers assemble into trimeric spikes in the viral membrane reviewed in [24, 25]. Note that two proteins are synthesized from the EBOV but not the MARV GP open reading frame (ORF). Thus, the main product generated is sGP, which is efficiently secreted from infected cells, while a fraction of the GP RNA is edited, resulting in the production of a message for full length GP [26, 27]. Expression of sGP was shown to reduce viral cytotoxicity and to increase production of progeny virions by infected cells [28]. Additional functions of sGP and other forms of soluble GP [29–32] are not well defined. However, it is conceivable that these proteins might function as decoys for antibodies and might modulate immune responses against the virus.

Upon release of the viral genome into the cellular lumen, a copy of the genomic RNA is synthesized by the viral L protein, and this RNA serves as template for the production of messenger RNAs and of progeny genomes. Budding of new virions is mainly driven by VP40, although other viral proteins contribute to this process [33]. VP40 contains an amino acid motif known as late domain (for its function late in the viral replication cycle) by which it recruits cellular proteins of the vacuolar protein sorting machinery to facilitate the release of viral particles from infected cells [34, 35].

40.2.3
Tropism

Filovirus outbreaks occur in remote locations, so only limited information on clinical and virological parameters of filovirus infection of humans is available. Valuable information on e.g. filovirus tropism and pathology was obtained from animal models (reviewed in [14]). While several animal models have been established, including infection of rodents, it is believed that infection of macaques reflects filovirus infection of humans most closely. Thus, infected macaques develop central clinical manifestations observed in humans, including rash, fever, bleeding and disseminated vascular coagulation and lymphopenia (reviewed in [14]).

A detailed analysis of EBOV infection of cynomologous macaques was recently reported [36, 37]. In the peripheral blood, the numbers of CD4 and CD8 positive lymphocytes and especially natural killer cells decrease early and throughout the infection, while the number of B cells remains relatively constant [37, 38]. Apoptosis of lymphocytes is readily detectable and several pro-apoptotic genes are expressed [37, 38]. Since T and B cells are not susceptible to filovirus infection, apoptosis of bystander lymphocytes is most probably the cause for the lymphopenia associated with EBOV infection. The infection of organs follows a certain order [37, 39–42]. Spleen and lymph nodes are early targets [37]. Subsequently, virus can be isolated from liver, lung and bone marrow and at the later stages infectious virus can also be detected in brain, testis, heart, pancreas and kidney [37]. Thus, filovirus HF is a multi-organ disease and spleen and lymph nodes are early targets.

40.2.4
Dendritic Cells are Major Targets of Ebolavirus

Which cell types are infected in these organs? Monocytes and macrophages are important targets of filovirus infection [43–47]. These cells are infected early and throughout the course of infection and might play a role in dissemination of the virus. Endothelial cells are positive for EBOV antigen at later stages of the infection. Destruction of these cells due to viral replication is only moderate in the macaque model [36] and possibly in EBOV infected humans [48], suggesting that endothelial cell infection and damage due to viral protein expression might not fully account for leakiness of blood vessels [36]. Besides macrophages and endothelial cells, a new target cell type was recently identified in EBOV infected macaques: DCs are early and sustained targets of EBOV infection [37]. Thus, antigen positive DCs can first be detected in cortical sinuses of inguinal lymph nodes and in axillary and mesenteric nodes [37]. The infected DCs are positive for dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN), a C-type lectin, which augments infection by filoviruses and a variety of other pathogens (discussed in detail below) (reviewed in [49, 50]). Subsequently, infected DCs are also detectable in the paracortex, located most often nearby high endothelial venules (HEV). In spleen, antigen positive DCs can be found in red pulp cords of Billroth and in marginal zones [37]. Budding virions can be occasionally detected in DCs positive for both DC-SIGN and viral

antigen, suggesting that infection of these cells can be productive. There are two populations of infected, DC-SIGN expressing DCs, of which only one exhibits dendritic morphology [37].

40.2.5
Filovirus Infection causes Aberrant Dendritic Cell Maturation

What are the functional consequences of filovirus infection of macrophages and DCs? Infected macrophages secrete cytokines and tissue factor (TF) in a dysregulated fashion (reviewed in [14, 16]). Cytokine release is believed to trigger vascular leakage and to indirectly promote bystander apoptosis of lymphocytes, while secretion of TF might induce coagulopathy (reviewed in [14, 16, 51]; see Fig. 40.1). Virus-induced release of mediators in general is thought to be responsible for much of the pathology of EBOV infection. The effects of filovirus infection of DCs have recently been examined [52, 53]. Two groups reported that DCs derived from blood monocytes (MDDC, monocyte derived dendritic cells) by treatment with GM-CSF and IL-4 are readily susceptible to EBOV and MARV infection. Viral replication in these cells is as efficient as in the Vero cell line, which is known to be highly per-

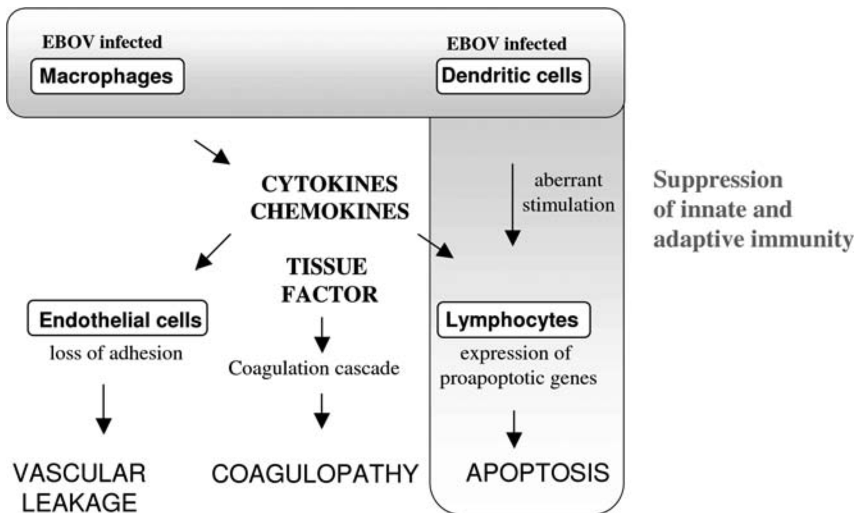

Fig. 40.1 Proposed key events in EBOV pathogenesis (adapted from [14, 16]). Macrophages and DCs are early targets of EBOV. Infected macrophages release mediators responsible for important aspects of EBOV pathogenesis. Thus, pro-inflammatory cytokines might trigger rearrangement of endothelial cells, resulting in vascular leakage, while tissue factor might activate the coagulation cascade, leading to disseminated intravascular coagulation. Finally, mediators produced from infected macrophages might induce expression of pro-apoptotic genes in lymphocytes. Aberrant stimulation of lymphocytes by infected DCs might also contribute to lymphocyte apoptosis. By infecting and functionally altering macrophages and DCs, EBOV suppresses key players of innate and adaptive immune defenses.

missive. When exposed to microbial antigens like lipopolysaccharides (LPS), DCs release cytokines, which induce an inflammatory response. However, filovirus infection of MDDCs or exposure of these cells to inactivated virus does not result in release of IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, RANTES, IFNα and TNFα [52, 53]. Nevertheless, EBOV infected cells are responsive to LPS treatment, indicating that filovirus infection does not interfere with the response to certain inflammatory stimuli. In contrast, LPS induced release of IFNα, which triggers the establishment of an antiviral state in cells, is fully suppressed in EBOV and partially suppressed in MARV infected DCs [52]. Suppression of IFNα release can also be observed with inactivated viral particles and is probably mediated by VP35 (see below) [52].

DC-mediated stimulation of T cells is crucial for the establishment of an adaptive immune response. However, incubation of DCs with infectious and inactivated EBOV and MARV reduces the capability of these cells to trigger proliferation of allogenic T cells in a mixed lymphocyte reaction (MLR) [52, 53], suggesting that filovirus infection fails to induce DC maturation, which is required for highly efficient T-cell stimulation. Indeed, exposure to inactivated or infectious filovirus particles induces abnormal DC maturation, characterized by inefficient or absent upregulation of co-stimulatory molecules like CD80 and CD86 [52, 53] and lack of downmodulation of CCR5 [52] (Fig. 40.1). Aberrant expression of these molecules can be rescued by exposing infected DCs to a potent stimulus like LPS [52, 53], suggesting that the infected cells remain fully responsive to potent external maturation stimuli.

40.2.6
Filoviral Protein(s) Suppress Dendritic Cell Maturation

From the studies described above it is not clear whether infected DCs simply fail to efficiently recognize the virus or if filovirus infection suppresses DC function. Analysis of virus-like particles (VLP) suggests that the latter is the case [54]. Co-expression of the filovirus GP and the matrix protein VP40 allows the formation and release of replication defective particles that in terms of morphology and antigenicity are very similar to infectious virions [55–57]. Vaccination of mice with VLPs triggers a strong antiviral immune response and protects animals from lethal EBOV infection [57]. VLPs, but not infectious or inactivated virus, might therefore be capable of activating DCs. Indeed, treatment of human or mouse DCs with VLPs triggers DC activation and maturation, as judged by expression of co-stimulatory markers, MHC I and MHC II, modulation of chemokine receptor expression, cytokine release and efficient induction of T-cell proliferation in a MLR [54, 57]. Therefore, a viral structural protein other than GP and VP40 seems to actively suppress DC maturation. VP35, which interferes with the action of interferon regulatory factor 3 [58, 59] and thus with cellular antiviral defenses, is an interesting candidate [54]. Characterization of VLPs containing specific viral proteins, apart from VP40 and GP, might provide an answer in short time.

In summary, filoviruses replicate in DCs and actively prevent normal maturation of these cells (Table 40.2). However, it is unclear which viral protein suppresses DC function. VP35 blocks IFNα release and might interfere with other DC responses to infection. Massive targeting of DCs and macrophages might promote viral dissemination to important target organs and might account for the absence of an effective antiviral immune response in most filovirus infected patients (Fig. 40.1).

Tab. 40.2 Functional characterization of infected MDDCs.

	IFNα, TNFα	*Co-stimulatory Markers/MHC*	*MLR*
EBOV, MARV	–	–	–
LV	–	–	–
DEN	+	+*	–
HTV	+	+	+

* Expression was observed on bystander but not on infected MDDCs [75].

40.3 Dengue Virus (DEN)

40.3.1 Epidemiology and Pathology

Filoviruses are notorious for their ability to cause disease with hemorrhagic manifestations, however, the vast majority of worldwide cases of viral HF is due to infection with DEN. In fact, two fifths of the world's population are at risk for DEN infection and about 50 million cases occur every year (reviewed in [4–6, 60]). DEN belongs to the flavivirus family (Table 40.1), which contains important emerging viruses, like West Nile virus. Several flaviviruses are transmitted by mosquitoes and ticks, and such viruses are termed arboviruses, for arthropode-borne viruses. The natural hosts for most arboviruses are birds and other small animals. In contrast, humans do not usually serve as reservoirs and are so-called "dead end" hosts, since intraspecies transmission and re-infection of mosquitoes is inefficient. DEN and yellow fever virus are exceptions from this rule, since both viruses have adapted to use humans and non-human primates as reservoir hosts. The mosquitoes *Aedes aegypti* and *Aedes albopictus* serve as vectors for DEN transmission and the former is responsible for most DEN transmission in urban environments (reviewed in [5, 6, 60]). While DEN fever (DF) epidemics were documented in the 19th century, the virus has experienced a massive spread during the last 50 years, which is closely paralleled by the spread of its vectors. Thus, while initially DF was mainly observed in South Asia, the virus is now also endemic/epidemic in India, China, Tahiti, Cuba, the Caribbean, Pacific Islands, Venezuela and Brazil (reviewed in [5, 6, 60]).

40.3.2
Replication

The DEN genome, a positive stranded RNA of 11 kB, encodes a single polyprotein, which is cleaved by viral and host cell proteases to yield ten mature polypeptides (Table 40.1). The genome is flanked by 5' and 3' untranslated regions, the 5' end is capped, but the 3' prime end lacks a poly A tail. The structural proteins comprise the capsid protein (C), the membrane protein M, which is generated as a precursor (prM) and the envelope protein (E) (reviewed in [61, 62]). Infection of target cells is mediated by E, which binds to cellular receptors that have not yet been well characterized, and mediates membrane fusion, most probably triggered by the low pH environment in endosomes. Upon its release into the cytoplasm the viral RNA is translated and negative stranded viral RNA is generated, which then serves as a template for the production of genomic and messenger RNA (reviewed in [61, 62]). Interactions of the structural proteins promote assembly of progeny virions in the endoplasmatic reticulum (ER), which are released from infected cells by exocytosis.

40.3.3
Dengue Hemorrhagic Fever

Four serotypes of DEN have been identified and infection with these viruses can cause DF, however, most infections do not induce clinical symptoms. DF is an acute, self-limiting illness, characterized by fever, myalgia, lymphadenopathy and rash and hardly ever produces a fatal outcome (reviewed in [4–6, 60]). Leukopenia and thrombocytopenia and altered levels of liver enzymes are common in DF. About 3% of DEN-infected individuals develop a severe form of the disease termed DEN HF (DHF). DF and DHF initially produce similar symptoms, however, when viremia decreases three to seven days after infection, DHF patients can exhibit plasma leakage, hemostatic abnormalities, coagulopathy and hemorrhage (reviewed in [4–6, 60]). Hemorrhages are often mild and maybe limited to petechia development. Vascular permeability can be severe and can cause a condition of shock, termed DEN shock syndrome (DHS). In the absence of medical attention DHF can be fatal in up to 44% of the cases, however, adequate treatment reduces mortality to as low as 0.2% (reviewed in [5]).

What are the reasons for development of DHF? Epidemiological studies demonstrated that a prior DEN infection predisposes individuals to development of DHF upon secondary DEN infection with a different serotype (reviewed in [60]). The humoral immune response might at least in part account for this predisposition, since antibodies raised against a certain DEN serotype can enhance infection by another serotype, a phenomenon termed antibody-dependent enhancement of infection (ADE) [63, 64]. ADE is particularly efficient when target cells express high level of Fc receptors, indicating that opsonized virions can infect target cells in a process that is augmented by antibody recognition by Fc receptors. Passive transfer of antibodies increased DEN viral loads in an animal model [65] and peak viral

load is thought to determine the risk for DHF [66–68], suggesting that ADE is relevant to DEN replication and pathogenesis (reviewed in [60]). The importance of pre-existing DEN specific antibodies is also highlighted by the observation that children born to DEN-infected mothers can develop DHF upon primary infection, probably due to the presence of maternal antibodies [69]. Besides B-cell immunity, the T-cell response in secondary DEN infection might also contribute to DHF. Thus, CD8+ T cells in patients with secondary DEN infection seem to recognize MHC presented peptides derived from the infecting virus with low efficiency – but might bind strongly to peptides derived from the virus which established the primary infection – and a substantial proportion of the T cells undergo apoptosis [70, 71]. Therefore, it has been suggested that presentation of suboptimal peptide antigens to T cells specific for DEN antigen present in the primary infection might lead to aberrant T-cell activation and mediator release, while preferential amplification of B cells specific for the DEN serotype responsible for the primary infection might result in the production of mainly non-neutralizing antibodies, which mediate ADE (reviewed in [60]).

40.3.4
Skin Dendritic Cells are Early Targets of Dengue Virus

Since DEN is transmitted to humans by mosquito bites it is conceivable that cells in the skin are potential targets. Langerhans cells (LC), DCs that reside in the top layer of the epidermis, are among the first cells to come into contact with the virus. These cells and MDDCs are indeed susceptible to DEN infection [72]. In fact, all DEN serotypes infect immature MDDCs with appreciable efficiency [72, 73], whereas mature MDDC [72], myeloid DCs [74] and plasmacytoid DC [74] isolated from blood are at least 10–100-fold less susceptible. Infection of immature MDDCs is productive [72, 73] and about 10-fold more efficient than that of macrophages [72], which are considered important targets of DEN. In contrast, monocytes, T and B cells and peripheral blood mononuclear cells are not readily permissive to DEN infection [72, 73]. Note that ADE can be observed with monocytes or certain cells lines, but not with MDDCs [66, 72], suggesting that virus entry into the latter cells is not facilitated by Fc receptors (see below). DEN infection of LCs was also demonstrated in cadaveric skin explants inoculated with virus and in skin samples from an individual immunized with attenuated DEN [72], confirming DEN infection of skin DCs *in vivo*. DCs might therefore be initial targets and a major source of virus during primary DEN infection, while in a secondary infection DEN replication in macrophages (enhanced by ADE) might be more prominent and might contribute to development of DHF [72].

40.3.5
Differential Effects of Dengue Virus on Infected and Bystander Dendritic Cells

Does DEN infection alter DC function? Several studies suggest that DEN infection of MDDCs triggers upregulation of CD40, CD80, CD86, MHC I, MHC II and

CD83 [73–76], all indicative of DC maturation. In agreement with these observations, cytokine production is also upregulated. Thus, DEN infection triggers the release of TNFα and IFNα [73, 75–77]. In DEN infected PBMCs, IFNα release is due to plasmacytoid DCs, suggesting that similar to other viral infections, these cells are the major source of IFNα in DEN infection [74]. In contrast to the robust DEN induced TNFα and IFNα release, the impact of DEN infection on IL-10 and IL-12 production by MDDC is less clear. Three groups reported slight to moderate induction of IL-12 production in infected MDDC [74–76] with overall output of IL-12 being enhanced in the presence of IFNγ [75], while one group failed to detect altered secretion of this cytokine upon DEN infection [73]. Similarly, no induction of IL10 production was observed upon DEN infection of MDDC in one study [75], while another study found induction of IL10 production [76], albeit to variable degrees. These discrepancies might be partially attributed to differences in the experimental conditions and the percentage of DEN positive cells in infected cultures. The latter might be of particular importance, since it has been suggested that DEN infection has differential effects on virus positive and bystander DCs [76]. Thus, one group observed upregulation of maturation markers only on bystander but not on infected DCs [76] and a second group also reported more pronounced effects with bystander cells, but detected upregulation of maturation markers on both infected cells and bystander cells [75]. A potential DEN-mediated block to DC maturation can be overcome by IFNγ but not by TNFα [75, 76], suggesting that negative modulation of DC maturation by DEN infection can be overcome by certain mediators. Thus, DEN infected MDDCs cultures release mediators like TNFα and IFNα, which can stimulate DC maturation, but DEN infected cells seem to fail to express robust levels of co-stimulatory and activation markers as well as MHC and might therefore be incapable of stimulating T cells with high efficiency.

Indeed, it has been reported that induction of T-cell proliferation by MDDCs in a MLR was reduced by DEN infection [76]. The reduction was dependent on the infectious dose, however, the correlation was not linear, suggesting a role for other factors, like cytokines [76]. Analysis of cytokine production in T cell/MDDC co-cultures revealed that DEN infection induced release of IL-10, which might be responsible for the decreased T-cell stimulation observed in the MLR reaction [76]. Apart of IL-10 release, induction IL-4 and IFNγ production was observed in DC/T-cell co-cultures, suggesting that DEN infected DCs might prime T cells to develop into a Th0 phenotype [78].

In summary, immature DCs are highly permissive to infection with all DEN serotypes. Infected DCs might not express sufficient amounts of molecules required for T-cell stimulation and might therefore fail to induce T-cell proliferation with high efficiency (Table 40.2). DEN inoculated MDDC cultures release TNFα and IFNα, which could promote maturation of bystander DCs. These cells might cross present antigen obtained from DCs undergoing apoptosis induced by DEN infection [75]. Release of IL-10, which is elevated in DHF patients [79], might negatively regulate antiviral immune responses. Thus, viral modulation of DC function combined with efficient infection of macrophages due to ADE, might be relevant features of DHF development.

40.4
Lassa Virus (LV)

LV, which is endemic in West Africa, causes disease in about 20% of infected individuals (reviewed in [80, 81]). It has been estimated that at least 59 million people are at risk of LV infection and up to 67 000 die from LV fever every year (numbers apply to Sierra Leone, Guinea and Nigeria) (reviewed in [80]). LV is transmitted from rats (*Mastomys natalensis*) to humans and can be spread by interhuman transmission. Manifestations of severe disease can include mucosal hemorrhages, hearing loss and pleural and pericardial effusion. No vaccines are currently available, however, ribavirin treatment clearly reduces death associated with LV infection (reviewed in [80]).

LV is an enveloped virus that contains two genomic, single stranded, ambisense RNA molecules (Table 40.1), which encode four viral proteins (reviewed in [81, 82]). The GP is synthesized as a precursor protein, which is cleaved in the ER or golgi compartment by a cellular protease. Dimers of the subunits GP1 and GP2 assemble into tetramers, which are incorporated into the viral membrane. Mature GP interacts with the LV receptor α-dystroglycan to drive fusion of the viral and the cellular membranes [83]. The viral RNA, which is associated with the nucleoprotein (NP), is transcribed and replicated by the L protein in the cytoplasm of infected cells. These processes are regulated by the Z protein, a small zinc binding protein known to interact with cellular factors and to be incorporated into virions (reviewed in [81, 82]).

Similar to infection with EBOV, it is believed that LV mainly targets macrophages and endothelial cells and that infection leads to an uncontrolled release of cytokines and to suppression of adaptive immune responses [53]. Both viruses have another feature in common, they target DCs and alteration of DC function might play a role in viral pathogenesis. Thus, immature MDDCs are highly permissive to LV infection [53, 84] and infected cells produce about 30-fold more progeny virions than infected macrophages [84]. Mature MDDCs are also productively infected by LV, however, viral replication is reduced compared with immature MDDC [84]. Infected DCs and macrophages remain viable without signs of apoptosis [84]. Therefore, both cell types might serve as early targets of LV infection and might be a continuous source of new virus throughout the course of LV infection. Infection of MDDC does not induce upregulation of activation/maturation markers (CD86, CD80, CD40, CD83, CD54, MHC I, MHC II, ILT3), does not interfere with phagocytosis, and does not trigger upregulation of cytokines (TNF-α, IL-1β, IL-6, IL10, IL-12, TGF-β, IFNγ and CD25) or chemokines (MIP-1α, MIP-1β and IL-8) [53, 84], clearly indicating that LV infection does not trigger DC maturation. LV infected DCs also fail to stimulate T-cell proliferation in a MLR [53], confirming lack of expression of molecules required for formation of a immunologic synapse between DCs and T cells (Table 40.2). Similarly, LV infection fails to activate macrophages and to induce release of soluble mediators by these cells [84]. Thus, it is unlikely that LV infected DCs or macrophages are relevant sources of cytokine production *in vivo* and these cells might not play a major role in cytokine induced vascular

permeability [84]. Moreover, LV interference with the antigen presenting function of DCs and macrophages might explain much of the virus-induced immunosuppression. In this context, it has been suggested that LV infection of immature DCs in the spleen might induce tolerance [84], a possible consequence of antigen presentation by immature DCs [85]. Expression of CCR5 and CCR7 is reduced in infected DCs and these cells exhibit diminished migration towards the chemokine MIP-1α [84]. LV infected immature DC might thus not be able to migrate into lymphoid tissue upon antigen uptake. Note that infected DCs remain fully responsive to maturation induced by LPS and soluble CD40L, indicating that LV might specifically suppress DC activation triggered by infection, but might not interfere with DC activation by other stimuli [84]. Interference of the Z protein of lymphocytic choriomeningitis virus (LCMV), another arenavirus, with host cell translation factors has been described [86] and it will be interesting to investigate if Z modulates DC function [84]. The observations described above would argue that infection of DCs and suppression of their ability to initiate immune responses might significantly contribute to LV replication and pathogenesis. An important role of DCs in the infection by other arenaviruses has indeed been documented [87, 88], and targeting of DCs was found to be important for immunosuppression (reviewed in [89]).

40.5 *Hantavirus* (HTV)

Infection with HTV, a member of the bunyavirus family (Table 40.1), can cause disease with hemorrhagic manifestations. Infection with old world HTVs (e.g. Hantaan, Seoul and Puumula) induces hemorrhagic fever with renal syndrome (HFRS), while infection with new world HTVs (e.g. *sin nombre*) results in HTV pulmonary syndrome (HPS) (reviewed in [90, 91]). Over 30 HTV serotypes have been identified, which differ in their capacities to induce disease in humans. The natural hosts of HTV are rodents, which are chronically infected but do not develop disease (reviewed in [90, 91]). Transmission of HTV from rodents to humans occurs via aerosolized excreta. HTV are enveloped viruses, which contain three single-stranded, negative sense, genomic RNAs, termed L, M and S. M encodes the viral GP, which mediates cellular entry by engaging β3 integrin (CD61) on the surface of target cells [92]. L harbors the information for a RNA dependent RNA-polymerase and S encodes the nucleocapsid protein (N), which is associated with the viral RNA. HTV is thought to mainly target macrophages and endothelial cells and replication seems to be noncytopathic [93–95]. Major aspects of HTV pathogenesis are believed to be due to a dysregulated immune response (reviewed in [96]). Strongly elevated numbers of activated CD8+ T cells are found in individuals with acute HFRS and might play a role in tissue damage [94, 97, 98].

HTV productively infects immature and mature MDDC as well as blood-derived DC and DC generated from CD34+ progenitor cells [94], indicating that DC might be readily permissive *in vivo*. As observed with macrophages and endothelial cells,

infection of MDDCs does not induce apoptosis [94]. HTV infection of MDDCs triggers maturation, as evidenced by upregulation of co-stimulatory markers and MHC I and II, reduced phagocytosis, release of TNFα and IFNα, but not IL-12, and efficient stimulation of T-cell proliferation in a MLR (Table 40.2) [94]. It is conceivable that HTV targets DCs in the lung early in the infection and employs these cells to ensure its dissemination in the host [94]. Mature, HTV positive DCs might stimulate CD8+T cells with extraordinarily high efficiency, which then attack and damage infected tissue. Finally, release of TNFα and IFNα might contribute to increased vascular permeability [94].

40.6 Filoviruses and DEN Engage DC-SIGN, a Lectin Expressed on DCs

40.6.1 DC-SIGN – a Portal for Pathogens

DCs express a variety of receptors, which recognize and take up specific antigens, among them toll like receptors and lectin receptors. The lectin DC-SIGN, which is expressed to high levels on DCs, binds and endocytoses mannosylated ligands for intracellular processing, ultimately resulting in presentation of peptide fragments by MHC molecules [99]. However, a large body of evidence suggests that a multitude of viral and nonviral pathogens specifically target DC-SIGN to facilitate their uptake into DCs (reviewed in [50]). These pathogens manage to escape degradation in lysosomes and subvert central features of DC biology, including migration into lymphoid tissue and establishment of immunological synapses with lymphocytes, to ensure their dissemination within the host (reviewed in [50]). The most prominent pathogen, which hijacks DCs via DC-SIGN and possibly structurally related molecules [100] is human immunodeficiency virus (HIV) [101]. Thus, HIV does not infect most types of DCs to appreciable levels, but virus pulsed cells facilitate infection of adjacent, susceptible T cells in a process termed infection in trans (or transmission) [102, 103]. Three lines of evidence suggest that DC-SIGN is involved in DC driven HIV transfer. First, transmission can be reduced by siRNA [104] or antibodies against DC-SIGN [101, 105, 106], however, variable blocking efficiencies were observed. Second, certain B cell lines engineered to express DC-SIGN transfer virus to susceptible cells in a DC-SIGN dependent manner [101, 107]. Third, DC-SIGN contributes modestly to HIV uptake by DCs in mucosal tissues analyzed *ex vivo* [105] and virus-pulsed cells can migrate along a chemokine gradient and transmit HIV to target cells, suggesting that HIV might exploit DCs as trojan horses to promote its transport into lymphoid tissue [101].

The interpretation of experiments assessing the contribution of DC-SIGN to HIV transmission with the help of specific blocking reagents is complicated by the role of DC-SIGN as a cell adhesion factor. Thus, DC-SIGN promotes intimate contact of DCs with T cells and endothelial cells by binding to ICAM-3 and ICAM-2, respectively [108, 109]. A close association between T cells and DCs, which involves

concentration of HIV particles and viral receptors at the cellular interface, is required for HIV transmission [110] and is also promoted by DC-SIGN [111]. Nevertheless, ICAM-3 expression on target cells was shown to be dispensable for efficient HIV transmission [112]. In summary, DC-SIGN is a potent attachment factor, that binds to high-mannose carbohydrates in the HIV envelope protein (Env) [113, 114] and facilitates HIV transmission to adjacent permissive cells. However, binding to DC-SIGN does not allow for productive infection [101], for which engagement of the viral receptors is indispensable. Transmission probably involves DC-SIGN mediated HIV endocytosis [115], followed by intracellular storage and, in the case of the virus-loaded cell coming into contact with a permissive cell, intracellular trafficking and concentration of virus particles at the cellular interface, which ultimately results in robust infection of the target cell [49].

Besides HIV Env, DC-SIGN interacts with a variety of appropriately glycosylated viral GPs, including the GPs of EBOV [116, 117], MARV [118] and DEN [119, 120]. LV GP does not engage DC-SIGN [117], but mediates infection of DCs by interacting with the cellular receptor α-dystroglycan [89]. A possible engagement of DC-SIGN by HTV has not been analyzed. However, the HTV receptor β3 integrin is expressed on DCs and might mediate viral entry into these cells [84]. In the following sections, the interaction between DC-SIGN and filoviruses and DEN will be discussed.

40.6.2
Does DC-SIGN Promote Filovirus Infection *in vivo*?

Expression of DC-SIGN on permissive cells like primary macrophages and 293 derived cell lines promotes infection by retroviral particles pseudotyped with the ZEBOV GP (so called pseudotypes) [116, 117] and by infectious ZEBOV [117], indicating that DC-SIGN expression could augment filovirus infection of target cells *in vivo*. Divergent results were reported on a possible role of DC-SIGN as a filovirus receptor upon expression on lymphoid cells, which are otherwise refractory to filovirus entry [121]. Thus, stable expression of DC-SIGN in the Jurkat T-cell line allowed entry of ZEBOV GP harboring pseudotypes [116], whereas expression of the lectin on primary T cells and on a B cell line did not render these cells permissive [117]. It is possible that Jurkat cells express low levels of filovirus receptor(s) and that DC-SIGN might augment receptor dependent entry. Indeed, DC-SIGN mediated enhancement of infection is particularly striking when low levels of receptors are expressed [122]. Ultimately, the role of DC-SIGN in filovirus entry can only be unambiguously clarified once the filovirus receptor(s) for entry into DC-SIGN negative cells has been identified and blocking reagents developed.

The role of DC-SIGN in filovirus interactions with DCs also awaits further investigation. Transmission of ZEBOV GP harboring pseudotypes from MDDCs to HeLa cells has been observed [116], but transmission efficiency seems to be relatively low. Most importantly, however, it is currently unclear if DC-SIGN plays a role in filovirus infection of DCs (Fig. 40.2). In this respect it is of interest that the GPs of EBOV subspecies engage DC-SIGN differentially, albeit DC-SIGN binding does not correlate with pathogenicity. Thus, ZEBOV infection can be fatal in up to 90%

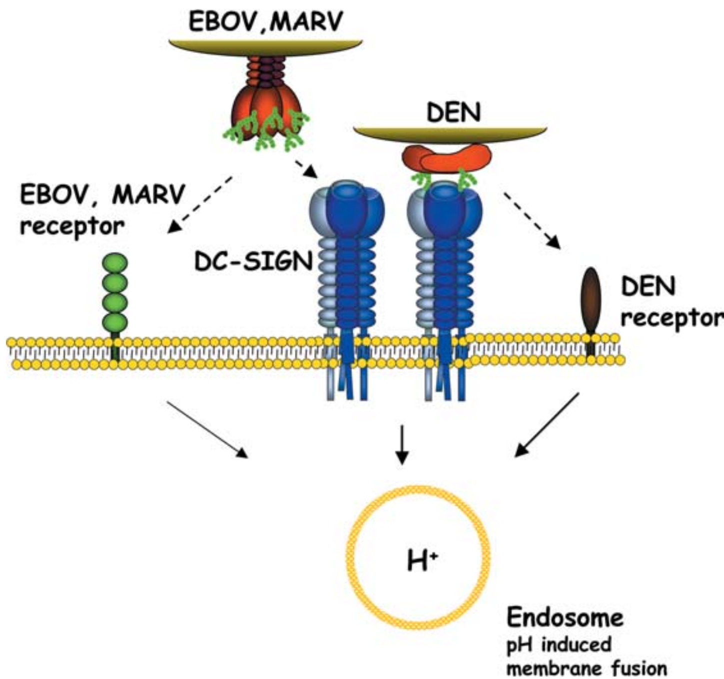

Fig. 40.2 Filovirus and DEN interactions with DCs. DEN engages DC-SIGN for entry into DCs. DC-SIGN might not facilitate infectious entry but augment DEN infection via a so far unidentified receptor. DC-SIGN expression on cell lines augments EBOV, MARV infection, however, it is unclear if DC-SIGN binding contributes to infection of DCs. Upon EBOV, MARV and DEN uptake into DCs via DC-SIGN and/or cellular receptors, virions are transported into endosomal vesicles, in which protonation triggers glycoprotein mediated membrane fusion.

of infected individuals and death associated with SEBOV infection is observed in up to 65% of the cases (reviewed in [16]), however, only ZEBOV but not SEBOV GP interacts efficiently with DC-SIGN [114]. Similarly, human infection with REBOV seems not to be associated with disease, while a single case of infection with ICEBOV was symptomatic with nonfatal outcome (reviewed in [16]), and REBOV but not ICEBOV engages DC-SIGN efficiently (S.P., unpublished observations and [116, 117]). The reasons for differential DC-SIGN engagement by the EBOV subspecies are incompletely understood, however, differential modification of the GPs with high-mannose carbohydrates is the most obvious explanation [114]. It remains to be determined, if differential DC-SIGN engagement is also observed with infectious EBOV and if DC-SIGN engagement correlates with the efficiency of DC infection. If such a correlation is established, DC-SIGN must be considered a target for antiviral intervention and vaccine delivery to DCs. Finally, it remains to be determined if soluble forms of GP bind to DCs via DC-SIGN and, if so, whether binding modulates DC function. The latter is questionable, since virion associated but not soluble EBOV GP fails to activate macrophages, indicating that adequate

presentation of GP inserted into the viral membrane is required for activation of APCs [123].

40.6.3
DC-SIGNR – a DC-SIGN-related Attachment Factor that might Concentrate Filoviruses in Liver and Lymph Nodes

Besides DC-SIGN, several other attachment factors might also impact filovirus tropism. Thus, human macrophage galactose- and *N*-acetylgalactosamine-specific C-type lectin (hMGL), which is expressed on DCs and macrophages, and asialoglycoprotein receptor (ASGPR), which is expressed on hepatocytes, might promote infection of these cells *in vivo* [114, 124, 125]. Moreover, DC-SIGNR (for DC-SIGN related, also termed L-SIGN, for liver SIGN), a lectin that shares 77% amino acid identity with DC-SIGN, might also capture viral particles and enhance infection [116–118]. DC-SIGNR is expressed on endothelial cells of liver and lymph node sinusoids [126, 127], both targets of EBOV infection [37]. Liver sinusoidal endothelial cells share several characteristics with DCs [128] and promote HBV transmission in an animal model [129], indicating that these cells might concentrate virus in the liver. It is therefore tempting to speculate that interactions with specific lectins rather than binding to the ubiquitously expressed viral receptor(s) might govern the cell and organ tropism of filoviruses [117]. However, analysis of lectin engagement by filoviruses in more relevant systems than stable cell lines will ultimately be required to define the contribution of specific lectins to viral spread.

40.6.4
Dengue Virus Targets Dendritic Cells via DC-SIGN

While the contribution of DC-SIGN to filovirus interactions with DCs remains to be determined, it is clear that DC-SIGN engagement efficiently promotes MDDC infection by DEN (Fig. 40.2) [119, 120]. Skin resident DCs are targeted by DEN upon transmission by mosquito vectors [72], therefore an important role of DC-SIGN in DEN infection must be considered. However, several questions remain to be addressed. First, how does DEN infect LCs? DEN infects LCs in tissue explants and in individuals vaccinated with an attenuated strain of DEN [72], suggesting that these cells are major targets. However, LCs do not express DC-SIGN [130] and it needs to be determined by which mechanism the virus enters these cells. DC-SIGN related lectin receptors, like the LC specific C type lectin Langerin, which binds to HIV Env [100], are interesting candidates. Alternatively, DEN might employ receptors it uses for infection of permissive cell lines.

Second, does DC-SIGN promote DEN attachment or entry? DEN infects a variety of DC-SIGN negative cell lines and this protein is thus not the only receptor for DEN, if it functions as a receptor at all. The latter can only be determined conclusively if a true nonpermissive cell line can be identified. The inefficient, but still detectable entry of DEN into the B cell line commonly used to assess DC-SIGN function (the THP and THP-DC-SIGN cell lines widely used to study DC-SIGN function are in fact B cells, most probably Raji B cells [119]), indicates that these cells

express DEN receptors other than DC-SIGN. It is therefore possible that DC-SIGN simply augments entry via these receptors (Fig. 40.2). On the other hand, it is conceivable that DEN might enter most cell types, as long as a mechanism for transport of virus particles into endosomes is provided, since the acidic environment in these compartments is probably sufficient to trigger the membrane fusion activity of the viral GP (reviewed in [131]). In agreement with a role of endosomal transport in DC-SIGN mediated enhancement of DEN infection, an endocytosis deficient variant of DC-SIGN exhibited reduced augmentation of infection [119, 120].

Third, do all DEN serotypes engage DC-SIGN for infection of DCs? One study found that the four DEN serotypes infect MDDCs efficiently, albeit minor differences were observed [120]. In agreement with a crucial role of DC-SIGN in MDDC infection, all DEN serotypes were also shown to engage DC-SIGN with comparable efficiency for infection of a B cell line [120]. However, another study reported that the DEN serotypes interact with DC-SIGN and infect MDDCs with differential efficiencies [119]. Thus, robust DC-SIGN usage and MDDC infection was observed only for DEN1 and DEN3 and correlated with the presence of an additional glycosylation site in the E proteins of these serotypes (the E proteins of DEN1 and DEN3 are glycosylated at N67 and N153, while the E proteins of DEN2 and DEN4 are only glycosylated at N67) [119]. Therefore, the glycosylation status of the E protein might determine DEN interactions with DC-SIGN. The type of carbohydrates attached to appropriate asparagine residues is controlled by the glycosylation machinery in the ER and golgi apparatus, which depends on the cellular background. Note that insect cells, when compared with human cells, preferentially modify GPs with high-mannose carbohydrates, the type of glycans recognized by DC-SIGN. DEN produced in insects cells might therefore bind to DC-SIGN more efficiently than virus produced in permissive human cells. Indeed, DC-SIGN engagement by Sindbis virus, another arbovirus, strongly depends on the virus producer cells, with infected mosquito cells releasing virions with a high capacity to bind to DC-SIGN [132]. Thus, DEN particles that establish the infection upon transmission from mosquito to humans will be especially adept in binding DC-SIGN, while DEN released from infected DCs might not engage DC-SIGN for efficient entry into target cells.

40.7 Conclusions

Filoviruses, DEN, LV and HTV productively infect MDDCs and at least for filoviruses and DEN clear evidence has been presented that these cells are major targets in infected individuals. MDDC infection by filoviruses, DEN, LV and HTV has differential effects, as assessed by cytokine release, upregulation of markers indicative for DC maturation, chemotaxis and T-cell stimulation in a MLR (Table 40.2). Filoviruses and LV do not induce any of these functions, while the effects of DEN infection can differ between virus infected and bystander cells. Filoviruses actively suppress MDDC maturation and indirect evidence for a specific suppression of DC maturation by LV and DEN has also been reported, however, the responsible

proteins remain to be identified. Filovirus and LV infection of DCs, which results in inhibition of efficient antigen presentation, might contribute to immunosuppression, a hallmark of severe disease induced by these viruses. Targeting of DCs by DEN might also interfere with the establishment of a coordinated immune response, but amplification of T cells and B cells specific for viral antigen present during primary DEN infection might be the major reason for the ineffective antiviral response in DHF patients. In contrast to filovirus, LV and DEN infection, targeting of MDDC by HTV leads to cell activation and maturation (Table 40.2), indicating that DCs should be capable of initiating a potent response to HTV infection. It has been suggested, however, that DCs presenting viral peptides might provide an excessive stimulus to T cells, and attack by over-activated $CD8^+$ T cells might explain some of the immunopathology in HTV infection. In summary, several HFVs can infect DCs and modulate their cellular functions, thereby promoting viral spread. Filoviruses and DEN might share DC-SIGN as a receptor for uptake into DCs and this lectin, which promotes DC infection by a variety of other pathogens, is an attractive candidate for antiviral intervention and might provide a means to direct vaccines to DCs (Fig. 40.2).

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Abbreviations

DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrin
DEN	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
EBOV	*Ebolavirus*
EHF	*Ebola* hemorrhagic fever
HF	hemorrhagic fever
HFRS	hemorrhagic fever with renal syndrome
HPS	hantavirus pulmonary syndrome
HTV	*Hantavirus*
ICAM	intercellular adhesion molecule
LF	*Lassa* fever
LV	Lassa virus
MARV	*Marburgvirus*
MHC	major histocompatibility complex
MHF	*Marburg* hemorrhagic fever
MLR	mixed lymphocyte reaction

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41
Dendritic Cells in Measles Virus Pathogenesis

Marion Abt, Nora Mueller and Sibylle Schneider-Schaulies

41.1
General Introduction

Measles virus (MV) is the causative agent of acute measles, a well-defined clinical entity normally contracted by children and young adults. The efficiency of its transmission is documented by the first known report of measles (in Egyptian hieroglyphics) which failed to recognize the infectious nature of the illness, and described it as a normal part of child growth and development. In spite of this high level of contagion, the virus causes acute infection only once in a lifetime of an individual, indicating that antiviral immune responses are efficiently generated and persisting. Whether immunity induced by live vaccination, as introduced more than 30 years ago, also persists for such periods in the absence of boosting by re-exposure has not been unequivocally resolved as yet. In spite of the availability of this efficient vaccine, more than 40 million cases of acute measles are annually reported worldwide with more than 800 000 cases of infant deaths. Although MV can and does cause pneumonia and encephalitis even in industrialized countries, most fatal cases develop in Third World countries on the basis of the transient, yet pronounced general immunosuppression induced by the virus during and for weeks after acute measles [1]. It was long before its first isolation and description, that MV was recognized as the first immunosuppressive pathogen. The term "anergy" was first coined by von Pirquet in 1908 [2] to describe the loss of DTH reactions to tuberculin in MV-infected individuals. Together with a high susceptibility to secondary infections, a marked lymphopenia and a rapid switch from an initial Th1 to a long lasting Th2 response the observations already made by von Pirquet are still considered as hallmarks of MV-induced immunosuppression *in vivo/ex vivo*. Dendritic cells (DCs) have an unique role in initiating and shaping immune responses, and so are regarded as central to the understanding of induction of MV-specific immunity but also of key aspects of immunosuppression brought about by this virus. Findings on both aspects will be reviewed here.

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41.2
The Virus: Structure and Genotypes

MV is the type species of the morbillivirus subgroup of the mononegavirales, and has a nonsegmented RNA genome of negative polarity (Fig. 41.1a). The pleomorphic virus particle consists of two structural subunits, the nucleocapsid core and the envelope (Fig. 41.1b). The genome tightly associated with the nucleocapsid protein (N) is the only target for the viral polymerase complex [the large (L) protein together with its cofactor, the phosphoprotein (P)], for transcription and replication. The highly basic C protein and the V protein both encoded within the P gene are dispensable for viral replication *in vitro* [3, 4], although there is evidence that they may play a role in viral pathogenesis *in vivo* [5–7]. The matrix protein M links the core to the host-cell-derived lipid envelope and interacts with the two glycosylated viral transmembrane proteins [8, 9]. The hemagglutinin protein (H) mediates attachment of virions to cellular surface proteins and provides a helper function for membrane fusion [10]. Viral and membrane fusion at neutral pH requires the MV glycoprotein complex consisting of an H tetramer and a fusion protein (F) trimer [11, 12]. The F protein is synthesized as a precursor (F_0) which is proteolytically activated into the disulfide-bond-linked F_1/F_2 heterodimer in the TransGolgi-compartment [13]. Its ability to mediate membrane fusion requires two conformational changes, the first of which is induced upon cleavage, while the second is triggered after receptor recognition and attachment of the H protein [14]. Although MV is serologically monotypic, the existence of co-circulating genotypes was re-

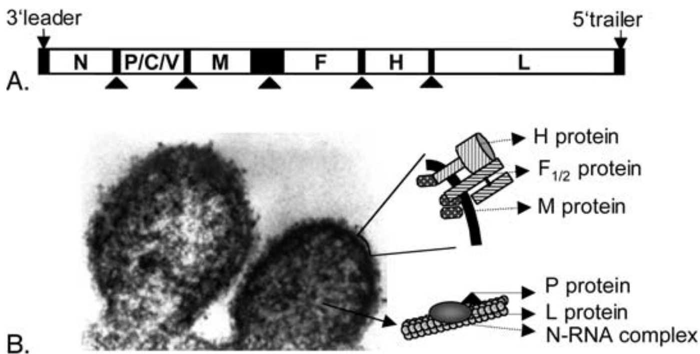

Fig. 41.1 (a) Schematic representation of the MV genome with transcription units indicated as white boxes. These are separated by intergenic regions (indicated as triangles) containing polyadenylation signals, an intervening trinucleotide and a transcription initiation site. The M and F genes are separated by a 1 kb-long noncoding region. Promotors for transcription and replication (3′ leader) and replication (5′ trailer) are located at the genomic termini; (b) Electron micrograph of two MV particles budding from the cell membrane. Enlargements show schematic representations of the two structural constituents, the envelope (containing the membrane proteins M, F and H) and the core (the genome encapsidated by the *N* protein and the polymerase complex consisting of the L (large or polymerase) protein and its cofactor, the P protein).

vealed by sequence analyses. Based on the N and H gene sequences, 8 clades (A to H), and within these clades, more than 20 genotypes were designated which reflect the genetic drift of MV strains [15–17]. The availability of an extensive database for wild-type viruses enabled molecular epidemiological studies tracing the source and transmission pathways of individual viruses or documenting interruption of indigenous transmission in some areas. MV wild-type strains do not differ in terms of pathogenicity or virulence, which are probably governed by host determinants such as immunocompetence. The basis for the attenuated phenotype of MV vaccine strains, which cluster within genotype A, is unknown. Most likely sequence motifs within the glycoprotein genes common to these strains are important determinants because these proteins essentially determine interaction with surface receptors and thereby cellular tropism and spread.

41.3
The Role of Entry Receptors in Measles Virus Pathogenesis

The first MV receptor identified was CD46 (membrane cofactor protein, MCP), a member of the complement regulatory proteins which is ubiquitously expressed on human nucleated cells [18, 19] (Table 41.1). Several splice variants of this protein are expressed in a tissue-specific manner, and all of them were found to support MV uptake after transgenic expression in some rodent cell lines [20]. Few amino acids within the MV H protein, including aa481, are critical for the interaction with an extended binding site spanning the two most membrane-distal short consensus repeats (SCR) of CD46 [21, 22]. In contrast, the “natural” ligands of CD46, the C3b/C4b complement components, require mainly SCR3 and 4 for binding [23]. As a consequence of infection, but also interaction with the H protein, CD46 is downregulated from the cell surface and this has, in agreement with the natural function of this molecule, been associated with an enhanced sensitivity to complement mediated lysis [24, 25]. Potential consequences of signaling elicited by MV CD46 ligation on cellular functions will be referred to below (see Section 41.4.1.2). High affinity binding to CD46 is, however, confined to attenuated vaccine strains and particular wild-type strains isolated on and adapted to grow on Vero cells, whereas these strains, but also lymphotropic MV wild-type viruses use CD150 (-SLAM; signaling lymphocyte activation molecule), a member of the CD2 subset of the Ig superfamily [26–29] (Table 41.1). This molecule is also downregulated by MV contact or infection with functional consequences being unknown as yet.

After primary infection in the respiratory tract, MV is most probably acquired by tissue resident macrophages or dendritic cells (DC) from the basolateral side of epithelial cells [9, 30] and transported to local lymphatic tissues. From there, after a first round of replication, MV spreads by a cell-associated viremia and MV-specific RNA and proteins can be detected in a small proportion of lymphocytes and monocytes during and for few days after the rash [31–33]. As a consequence of its viremic spread, MV infects a number of cell types, particularly within the endothelial and epithelial cell layers. The onset of virus specific immune responses is

Tab. 41.1 Cellular surface molecules on DCs interacting with MV proteins.

	CD46	***CD150***	***TLR2***	***FcγRII (CD32)***
Superfamily	Regulators of complement activity	Immunoglobulin	Pattern recognition receptor	Immunoglobulin
Ectodomain	4 short consensus repeats/ variable number of serine-threonine-proline rich repeats	One variable, one constant Ig-domain	Repetitive leucin-rich repeats	2 similar Ig variable domains
Tissue distribution	All nucleated human cells	Activated lymphocytes, monocyte/macrophages, DCs	High on professional APCs	Lymphocytes, monocyte/ macrophages, DCs, NK cells and neutrophils
Cellular function	Protection from complement mediated lysis	Regulation of lymphocyte function	Activation of APCs	Binding of Fc receptors
Endogenous ligand	C3b/C4b complement components	Self ligand	none?	Ig Fcγ
MV-Interaction	Mostly vaccine and adapted strains (H protein)	All MV strains (H protein)	MV wild-type strains (H protein?)	MV N protein
Inhibition with specific antibodies	Yes	Yes	yes	Yes
Regulation of host cell functions	Yes	Yes	yes	Yes
MV uptake in transfected CHO cells	yes	yes	no	no

marked by the infiltration of mononuclear cells into local areas of virus replication and the appearance of antiviral antibodies and virus-specific T cells in the blood. Activation of virus-specific T cells is documented by soluble CD4, CD8, IL-2R and β2 microglobulin and an initial Th1 cytokine profile which switches to a Th2 type as indicated by a rise in IL-4 plasma levels (reviewed in [34]). Whether a Th1 response is less efficient after vaccination than after natural infection, has not been clearly resolved as yet [35–37]. While MV-specific immunity is efficiently induced in the course of acute measles and after vaccination, there is a generalized suppression of immune responses to secondary infections in acute measles. Characteristically, a marked lymphopenia affecting mainly the T-cell population and a loss of delayed type hypersensitivity reactions are seen. Though there is evidence from one study that B-cell numbers are also reduced [38], it is unknown whether is a direct effect or rather relates to depletion or T-cell dysfunction. The impaired proliferative response of lymphocytes to polyclonal and antigen-specific stimulation *ex vivo* is considered as one of the major hallmarks of MV-induced immunosuppression [39, 40]. This can be seen for several weeks after acute measles, and also, albeit to a moderate extent, after vaccination [41]. In addition, a cytokine imbalance as seen by a predominant Th2 response and thereby suppression of cellular immunity has also been linked to immunosuppression [34].

The tropism of MV during natural infection is not fully explained by its usage of the receptors known. Given its broad cell tropism particularly late in infection CD46 would be an ideal candidate entry receptor. However, most wild-type strains rely almost exclusively on CD150 [29], the expression of which is confined to cells of the hematopoietic system. There, the molecule is expressed on activated B cells, activated and memory T cells (including activated regulatory T cells [42]) and immature thymocytes [43], activated monocytes and on maturing DCs [44, 45]. Other target cells identified *in vivo* such as endothelial cells, epithelial cells and, in persistent brain infections, neural and neuronal cells, do not express CD150. It is thus still not resolved whether MV wild-type strains may be able to use CD46, albeit with low efficiency, for entry, or if other, as yet unknown receptors are present. In support of the latter assumption, wild-type MV infection of endothelial cells *in vitro* could not be blocked by CD46 or CD150-specific antibodies [46].

41.4 Dendritic Cells in Measles Virus Pathogenesis

Although it is very likely that DCs are targets of MV, involvement of these cells *in vivo* has not yet been documented to occur in natural infection. In humans, this will probably not be feasible at all, assuming that infection of these cells might occur early after exposure. In experimentally infected non-human primates, infection of DCs has not been addressed directly. Association of MV with FDC has been documented in these animals [47]. Whether FDC could be considered as long term repositories for MV is currently unknown**.** The other animal model allowing for MV infection via the respiratory tract involves cotton rats (*Sigmodon hispidus*). In these

animals, immunosuppression as evidenced by impaired proliferation of lymphocytes *ex vivo* is apparent [48, 49]. Documentation of DC infection by MV has not yet been possible, because of the lack of reagents and protocols. In CD46 transgenic mice, splenic DCs were found to be activated and infected by the attenuated MV ED strain only after depletion of monocyte/macrophages [50, 51]. The relevance of this finding is not clear since this study was confined to the ED strain and these mice were also deficient for the type I IFN receptor. More recently, MV antigens were detected in very limited number of splenic CD11c$^+$ DCs after intravenous MV wild-type infection of mice transgenic for CD150 driven by the CD11c promotor with an MV wild-type strain [52]. In this system, DCs and a limited amount of macrophages were the only cells the virus could access, and thus potential infection of other cell types expressing CD150 in humans could not be evaluated. Thus, the vast majority of findings related to the role of DCs in MV pathogenesis and immunomodulation relies on *in vitro* findings obtained in pure DC or DC/T-cell co-cultures.

41.4.1
Measles Virus Interaction with Receptors on Dendritic Cells and Functional Consequences

41.4.1.1 Interaction with Surface Receptors

Langerhans cells and DCs isolated from peripheral blood or generated *in vitro* from monocytes or CD34$^+$ precursor cells could be infected with both wild-type and vaccine strains as documented by the accumulation of viral proteins [53–60]. Since CD46 is expressed on all these populations, infection with CD46-adapted strains is not surprising. Interestingly, however, MV wild-type strains and recombinant MVs expressing wild-type derived H proteins (which rely on CD150 for entry) revealed a particular tropism for DCs *in vitro* and probably also *in vivo* since they replicated preferentially in secondary lymphoid tissues of experimentally infected cotton rats [57, 61]. The importance of CD150 for DC infection was strongly supported by the requirement of this molecule for MV wild-type infection in CD11c$^+$ DCs from transgenic mice (see above), enhanced susceptibility of monocytes to MV infection after activation induced CD150 expression [45] or the inhibitory effect of CD150 antibodies on DC fusion [57]. While expression of CD150 was detected after LPS- or CD40L-driven maturation of DCs, and was found to be further upregulated by IL-1β [44, 62], DCs generated *in vitro* can also express this molecule to a moderate extent and thereby infection of wild-type MV could be mediated. CD150 can, however, also be induced by MV wild-type strains themselves. This may relate to the ability of these strains to induce CD150 expression in monocytes (and probably in DCs) since they can act as Toll-like receptor 2 (TLR2), but not, as shown for respiratory syncytial virus, TLR4 agonists [45, 63, 64] (Table 41.1). Interestingly, the TLR2 agonistic activity of MV strains seems to inversely correlate with their ability to interact with CD46. Thus, a recombinant MV expressing a wild-type H protein with aa481 reversed to that found in attenuated strains failed to activate TLR2 signaling [63]. Although the TLR2/CD14 complex does not support MV entry into target cells, interaction of MV wild-type strains may, besides CD150 induction, well

contribute to MV-induced maturation of immature DCs (see Section 41.4.1.2). Preferential MV entry into DCs does, however, apparently also involve other, uncharacterized mechanisms. Thus, the preferential tropism of wild-type H protein expressing MV strains did not correlate with enhanced binding to these cells but rather to an enhanced uptake of virus which could only partially be blocked by CD150-specific antibodies [57]. It is therefore likely that surface molecules should exist, which may not directly promote, but rather enhance MV uptake into DCs. Surprisingly, the MV N protein also seems to directly interact with the surface of DCs via the FcγRII [65] (Table 41.1), and recently, cell surface delivery of the N protein and its subsequent release has been shown to occur in infected cells [66].

41.4.1.2 Functional Consequences of Measles Virus Surface Interaction with Dendritic Cells

MV wild-type dependent activation of TLR2 presumably accounts for the induction of CD150, CD86 and cytokines such as IL-6, IL-12p40^{-}, and IL-1α/β [63, 67]. Other consequences of CD150 ligation by MV on DCs remain, however, speculative. Signaling properties of this molecules have been mainly studied after ligation with antibodies on lymphocytes. CD150 was found to favor CD95-mediated apoptosis in some B- and T-cell lines [68], but also to act as a co-stimulatory molecule on T cells, strongly enhancing IFN-γ production and thereby a Th1 response [69–71] (for review see [72]). Strikingly, studies in CD150$^{-/-}$ mice fail to support a critical role of this molecule in IFN-γ production in T cells but rather indicated that CD150 may enhance TCR stimulated IL-4 release. This study provided also evidence that CD150 may modulate TLR4 but not TLR2 or TLR9 signaling in macrophages. Thus, LPS-stimulated production of IL-12, TNF-α and NO were diminished and that of IL-6 was enhanced in the absence of CD150 [73]. In DCs the role of CD150 ligation either by antibodies or MV has not been addressed as yet. CD46 has also signaling properties the relevance for MV pathogenesis of which has to be considered in the light of mainly attenuated strains interacting with this molecule. Thus, CD3/CD46 co-ligation with antibodies is associated with T-cell activation, actin reorganization and GTP loading of Rho family GTPases [74, 75]. Moreover, murine T cells transgenic for CD46 isoforms differing in their cytoplasmic tail revealed impaired or, respectively, increased contact hypersensitivity responses with CD8 T-cell cytotoxicity, CD4^{+} T-cell proliferation and IL-2 versus IL-10 production being also differentially affected [76]. More recently, co-ligation of CD3/CD46 was found to trigger differentiation of regulatory T cells [77]. In macrophages, ligation of CD46 by MV was associated with enhancement of IFN-γ-stimulated NO production [78]. The effect of CD46 ligation on IL-12p70 production by professional APCs including DCs remains controversial and will be discussed below (see Section 41.4.3). Although the basis has not been defined in molecular terms, APC isolated from CD46 transgenic mice pulsed with UV-inactivated attenuated MV fail to stimulate contact-allergen specific proliferation of both CD4 and CD8 T cells [65]. In contrast, transgenic expression of CD46 on murine B cells and DCs allowed for capture of MV particles resulting in highly efficient presentation of viral antigens

to MHC class II restricted T-cell hybridomas [79, 80]. In agreement with the latter findings, monocytes infected with an attenuated MV strain efficiently presented MV, but not unrelated antigens to T cells in a MHC class II restricted manner [81]. As also seen for CD46, pulsing of wild-type, but not FcγRII deficient mice with N protein led to a failure of APC to stimulate contact-allergen specific T-cell proliferation [65].

41.4.2 Impact of Measles Virus on Dendritic Cell Viability and Maturation

As indicated above, DCs are infectable with both attenuated and wild-type MV strains, albeit with different kinetics. In addition to their preferential uptake wild-type strains replicate faster in DCs than attenuated strains [57, 58] indicating that MV proteins other than the glycoproteins favor intracellular replication in DCs as well. Although MV replicates in DCs immature or LPS matured at the infection time as indicated by accumulation of viral proteins, virus release is low from immature and almost absent from mature DCs [54, 59]. Apparently, the differentiation stage of DCs imposes particular constraints on MV release. This has also been described in differentiating monocytes/macrophages [82]. MV protein production and virus release was found to be enhanced in the presence of activated T cells, and this was related to CD40 ligation [54, 59]. In contrast, no impact on viral replication or syncytium formation upon CD40 ligation could be seen in another study [83]. Due to the accumulation of the viral glycoprotein complex on the surface of infected DCs, DC–DC fusion, as well as DC fusion with co-cultured T cells, was observed [55]. In this and a more recent study [52], apoptosis did occur in DCs cultures late after infection. When infected DCs were stabilized with a peptide inhibiting cellular fusion, DCs remained viable for at least 48 h [53, 56, 58]. DCs immature at the onset of infection rapidly undergo a phenotypic maturation as indicated by upregulation of MHC class I and II molecules, CD40 and co-stimulatory molecules such as CD80, CD83 and CD86 [53, 58, 59]. Maturation of DCs by MV is also indicated by cytokine production. Transcripts specific for IL-12p35, IL-12p40, IL-10, IL-1a/b, IL-1RA, and IL-6 were induced after infection of monocyte-derived DCs with attenuated MV strains [59] while production of IL-12p70 and IL10 on protein level was not detectable after infection with both wild-type or attenuated strains [83]. In contrast, IL-10 was detected on both mRNA and protein level in MV ED infected DCs generated from $CD34^+$ progenitor cells [53]. Moreover, again on the transcriptional level, induction of IL-23p19 by attenuated, but not wild-type strains and IL-18 by all strains was documented in DCs [83]. Mechanisms accounting for induction of cytokine specific transcripts particularly by attenuated MV strains which cannot activate TLR2 signaling are as yet unknown. Infection with these strains causes production of type I IFN from DCs of either origin which has partially related to DC maturation at least in terms of upregulation of CD80 and CD86, but also TRAIL [53, 56, 84]. Interestingly, induction of TLR3 by attenuated MV strains was also dependent on type I IFN as produced during infection [85]. Unlike for influenza virus [86], induction of the type I inducible MxA protein by these strains did not pre-

vent viral replication indicating that this protein might either act in a cell type specific manner in controlling MV infection [87, 88] or was not produced to protective levels. MV wild-type strains failed to induce type I IFN and were found insensitive to the antiviral activity of this cytokine in PBMC cultures [89]. Whether or to what extent this also applies to MV wild-type infection of DCs has not been studied as yet. This aspect appears particularly relevant since type I IFN-dependent induction of a cytolytic activity of DCs has been described [90, 91] (see Section 41.4.4).

41.4.3 Impact of Measles Virus on External Maturation/Stimulation Signals in Dendritic Cells

External signals such as those triggered by TLRs, cytokines and CD40 are important for full activation, mobilization and induction of terminal maturation of DCs. The ability of MV-infected DCs to migrate in response to chemokines has not been addressed as yet. There is, however, evidence that chemotactic responses at least to MIP3α are impaired [53]. Apparently MV may modulate TLR signaling. This is evident from studies done in monocytes where LPS- or SACS-stimulated production of IL-12p70 was strongly inhibited after exposure to MV [92]. This inhibition relied on crosslinking of CD46 as it also occurred with CD46-specific antibodies and C3b/C4b complement components. Long- term suppression of IL-12 release after SACS-stimulation of PBMCs isolated from measles patients supports an impaired production of this cytokine [93]. In contrast, early after infection the ability of DCs isolated from peripheral blood of healthy donors to produce bioactive IL-12 in response to LPS or SACS was not impaired by infection with MV wild-type or vaccine strains [58]. The mechanism by which non-CD46 interacting MV wild-type strains inhibit TLR-stimulated IL-12 production late after infection is unknown. Possibly, lack of stimulated IL-12 production *in vitro* may relate to an enhanced susceptibility of DCs to apoptosis *ex vivo* as seen in CD150 transgenic mice [52] rather than to interference with TLR signaling. It has not been addressed as yet whether T-cell attracting chemokines are released from MV infected DCs spontaneously or after stimulation nor has their ability to conjugate T cells evaluated directly. Ligation of CD40 by activated T cells or antibodies resulted in an enhancement of DC infection suggesting that CD40 signaling pathways activate viral replication to a certain extent [59]. With regard to further modulations of CD40 ligation by MV, controversial results were obtained. Interference with CD40 signaling was documented as indicated by downregulation of tyrosine-phosphorylation of cellular proteins, of IL-12p40 transcripts and IL12p70 production, induction of IL-10 transcripts and failure of MV-infected DCs to drive proliferation of $CD8^+$ T cells after CD40 ligation [59]. In contrast, enhanced production of IL-12p70 and IL-10 at the protein level occurred after CD40 ligation of MV-infected DCs as well [53], and we have recently found that production of these two cytokines after CD40L mediated activation from DCs infected with vaccine or wild-type MV was almost equivalent to that seen in mock treated cells [83]. Thus, MV-mediated modulation of CD40 signaling, its targets and consequences are far from being understood and require reevaluation

with virus strain, timing of stimulation and probably source of DCs being critical components.

41.4.4
Impact of Dendritic Cells Measles Virus Infection on T-cell Viability, Activation and Expansion

Although production of infectious virus from DCs is limited, transmission of MV to T cells has been reported which then are recruited into syncytia or die from apoptosis [54, 55]. Production of functional TRAIL from MV-infected DCs has been reported [84], and, moreover, activated T cells can induce apoptosis of infected DCs via CD95 ligation [52, 94]. Both settings would result in loss of DCs and T cells. Particularly T-cell lymphopenia in the peripheral blood compartment, to which aberrant homing of these cells might also contribute [95] is commonly seen in measles. There is, however, ample evidence that in spite of maturation induced upon virus encounter, MV-infected DCs fail to stimulate expansion of allogenic T cells independent of the induction of apoptosis [53–56, 58–60]. As outlined above (Section 41.4.3), it is possible that aberrant terminal maturation of these DCs contributes to this phenomenon at least for $CD8^+$ T cells. Since allogenic T-cell proliferation can not be induced by mitogenic signals in the presence of MV-infected DCs, it is apparent that these cells actively provide an inhibitory signal. Soluble mediators potentially involved in this suppression have not been identified, and a contribution of type I IFN or IL-10 has been ruled out [53]. Moreover, T-cell inhibition by MV-infection is contact dependent [53, 56] strongly suggesting surface molecules as being required for negative signaling to T cells.

Cell surface delivery of the N protein was described in a variety of cell types [66] probably also including DCs. As a result of FcγRII interaction, antibody production from B cells and DC function are impaired. Whether and to what extent surface expression of N protein on DCs might contribute to negative signaling to contacting T cells remains to be resolved. It has been demonstrated that soluble N protein interacts via its C-terminal domain with an unknown receptor different from FcγRII and can cause proliferative inhibition of thymic epithelial cells [96]. The relevance of this interaction for DC-mediated T-cell proliferation particularly for MV wild-type strains is unclear since (1) expression of this N protein receptor on T cells required prior activation; (2) the C-terminal domain of the N protein is the most variable region within MV strains, and only the N protein of an attenuated strain was tested; and (3) inhibition of T-cell proliferation by soluble N protein was not directly evaluated [96]. In contrast, there is direct evidence that the MV glycoprotein complex as expressed on infected cells including DCs mediates inhibition of T-cell proliferation in a contact dependent manner [53, 56, 58, 65, 76]. Thus, the inhibitory activity of DCs directly correlates with the expression levels of these proteins, and T-cell proliferation is not stimulated by uninfected DC in the presence of UV-inactivated MV. Furthermore, DC infected with a recombinant MV expressing the G protein of vesicular stomatitis virus instead of the MV glycoprotein, reveal a high allostimulatory activity and do not inhibit mitogen-driven T-cell proliferation [56].

Further supporting the importance of the MV gp complex in T-cell inhibition, the latter was efficiently prevented in the presence of MV F- or H-specific antibodies [53]. Expression of the MV F/H complex on virus particles or infected or transfected cells was found necessary and sufficient to induce arrest of stimulated T-cell proliferation *in vitro* and *ex vivo* [48, 97, 98]. This occurred independently of apoptosis, soluble mediators, complex glycosylation and fusogenic activity of the effector F/H complex, but required proteolytic processing of the F protein [98, 99]. In contrast to the observations made in CD46 transgenic mice [65], induction of T-cell proliferative arrest occurred independently of CD46 or CD150 [26, 97, 100]. After surface contact with the effector complex T cells accumulate in the late G_1-phase as characterized by typical restrictions of cyclin-dependent kinases and their inhibitor, $p27^{KIP1}$ [100–102]. Insight into the signaling pathways targeted by the viral F/H effector complex came from our studies where both IL-2- and CD3/CD28-dependent activation of the phosphatidyl-inositol-3-kinase (PI3K) pathway was found to be almost completely abolished both *in vitro* and in lymph-node cells of MV-infected cotton rats *ex vivo*. In support of the importance of this interference with T-cell unresponsiveness, the latter could be efficiently prevented upon expression of a constitutively active Akt kinase, which is one of the most important PI3K substrates in T cells [103]. Lack of CD3/CD28-stimulated PI3K activation after MV contact resulted in inefficient recruitment of the Akt kinase and the guanosine exchange factor Vav to lipid rafts. Interestingly, partitioning of the PI3K regulatory subunit, p85, to these domains was also abolished. This was not associated with inefficient tyrosine-phosphorylation of p85, but rather with the failure of the CD3/CD28 signal to induce degradation of its inhibitor, Cbl-b. In addition of inhibiting the recruitment of important components of the TCR signalosome to the lipid rafts where signaling needs to be initiated, MV was found to bind to lipid rafts at the cell surface [104]. The receptor is as yet unknown, but obviously different from CD46 which is a non-raft molecule and not firmly bound by wild-type strains, and CD150 which is not expressed on nonactivated T cells.

41.5 Conclusions and Perspectives

Given the role of DCs to induce and shape immune responses, it is evident that interaction with these cells is central to immunomodulation by MV although their interaction has not been extensively studied *in vivo*. From *in vitro* data (which are summarized in Fig. 41.2) it is clear that modulations of DC viability and function are induced by MV independently or as a result of infection with the pattern and efficiency partially dependent on the virus strain used. DCs might serve as primary target cells during infection and receive their maturation signals, be it by TLR signaling, interferon production or other as yet unknown stimuli. MV can enter into DCs via its known receptors, and these support viral replication. It has, however, not been defined as yet whether other modes of uptake such as endocytosis (for instance via DC-SIGN as for other viruses [105, 106] are also operative. If the latter

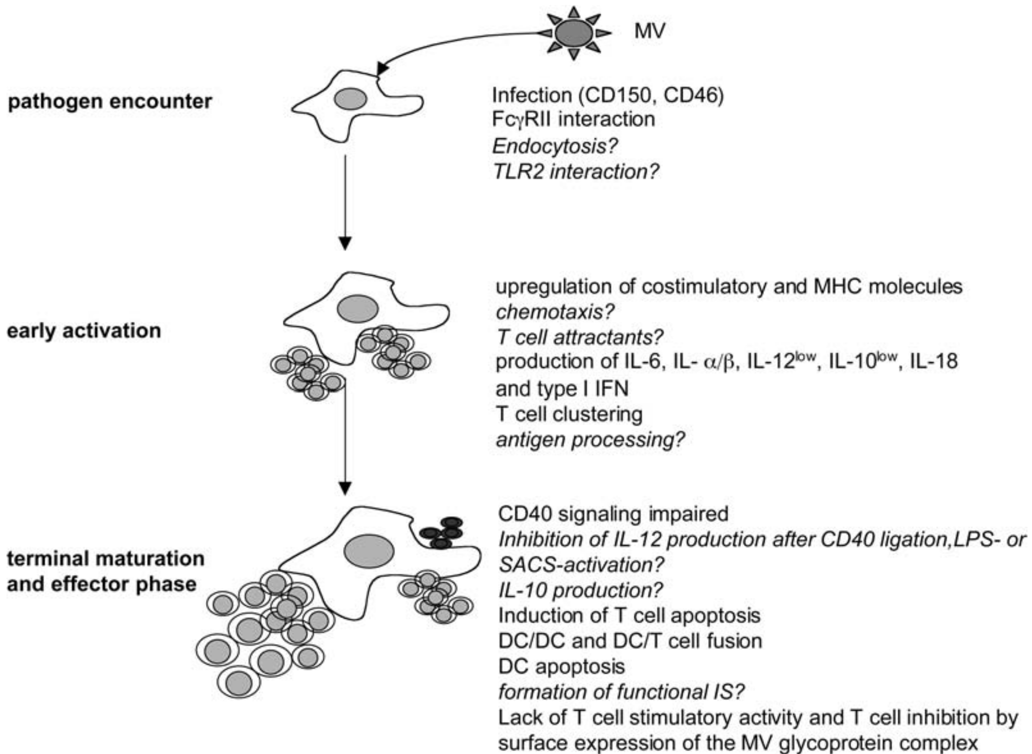

Fig. 41.2 Modulation of DC maturation and functions by MV. Consequences of MV interactions with DCs are indicated on the right hand side of the diagram. Those which have not been analyzed or for which findings are still conflicting are indicated in italics. Apoptotic T cells (lowest row of the diagram) are indicated in dark gray.

were to be true, or early after infection, processing of MV proteins for subsequent MHC loading and presentation most probably predominates since MV-specific antigens were found to be efficiently presented both *in vitro* and *in vivo*, and this results in the efficient induction of MV-specific immune responses. There are some *in vitro* observations which support the assumption that initial DC activation and T-cell stimulatory activity are not grossly affected early after MV encounter. Thus, these cells retain their phagocytic activity, reveal a normal phenotypic and functional maturation pattern, and are perfectly able to stimulate allogenic T-cell proliferation *in vitro* [56, 83]. Although their potential to migrate along chemokine gradients and to attract naïve T cells has not been studied in detail, the efficiency of induction of an MV-specific immunity strongly argues that they reach the secondary lymphatic tissues. If and how efficient this mobilization occurs will have to clarified *in situ* in experimentally infected non-human primates. These will also be instrumental in evaluating which of the phenomena linked to MV-induced immunosuppression suggested by *in vitro* experimentation are likely to contribute preferen-

tially to immunosuppression *in vivo*. Loss of DC or T cells by fusion most probably does not occur to a large extent since giant cells were very infrequently seen in lymph node material of rhesus macaques [47]. One study describes giant cell formation (called Warthin-Finkeldes cells) in hyperplasic lymphoid tissue in a measles patient at autopsy [107]. This was, however, not studied in uncomplicated measles, and, furthermore, evidence for MV in these lesions was provided only by immunohistology while MV could not be detected by electron microscopy [107]. Thus, the relevance of these findings is unclear. Rather, necrotic areas were noted in lymphoid tissues of infected rhesus monkeys [108]. Incomplete T-cell activation or active inhibition of T-cell expansion may predominate late in MV infection of DCs. Then, apoptosis, inability to respond to external maturation signals and, in addition, accumulation of viral proteins on the cell surface may collectively act in T-cell silencing or killing via TRAIL. The latter might be an important parameter in T-cell lymphopenia, but certainly does not explain why healthy uninfected peripheral T cells cannot proliferate upon exogenous stimulation. For this, direct negative signaling of infected DCs to scanning T cells, by expression of MV N or glycoproteins, is an attractive hypothesis. Obviously, T cells do not die as a consequence of this inhibitory signal and, at least *in vitro*, recover and regain their mitogenic response. In agreement with this, recall responses which are suppressed during and after measles, return to normal also *in vivo*. Given the heterogeneity of results obtained on cytokine production from MV-infected DCs there are only theoretical considerations on the type of T-cell responses induced by these cells. Due to the inhibitory effect of MV-infected DCs on T-cell expansion and their ability to fuse with these cells, long term co-cultures required to study T-cell differentiation patterns are not feasible. It has, however, recently been shown that soluble mediators are produced from DCs infected with either wild-type or vaccine MV which efficiently trigger expansion of IFN-γ-producing T cells from $CD45RO^-$ T-cell populations. Interestingly, this did also occur when uninfected DCs used APCs in this assay were generated in a way that they would normally support expansion of IL-4 producing T cells [83]. It is also possible that particularly those DCs infected with attenuated strains, may induce differentiation of regulatory T cells. Induction of these cells has been shown to occur after CD3/CD46 cross-ligation [77], and T cells contacting DCs infected with attenuated strains are expressing both MHC molecules and H proteins interacting with CD46. Moreover, these cells also produce type I IFN which has also been involved in T*reg* induction [109]. It is certainly within the near future that this hypothesis will be experimentally addressed and that the frequency and potential activation of this particular T-cell subset will be evaluated *ex vivo*.

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Abbreviations

APC	antigen-presenting cell
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrin
DTH	delayed typed hypersensitivity
ED	measles virus Edmonston strain
FDC	follicular dendritic cell
gp	glycoprotein
IS	immunological synapse
LPS	lipopolysaccharide
MV	measles virus
SACS	*Staphylococcus aureus* Cowan strain
TCR	T-cell receptor
TLR	Toll-like receptor
WTF	measles virus wild-type strain

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42
Dendritic Cells and *Herpes Simplex Virus Type 1*

Alexander T. Prechtel and Alexander Steinkasserer

42.1
The *Herpes Simplex Virus Type 1*

42.1.1
A Well-known Plaque for Centuries

"O'er ladies lips, who straight on kisses dream,
which oft the angry Mab with blisters plaques,
because their breaths with sweetmeats tainted are."
William Shakespeare, *"Romeo and Juliet"* 1595

These lines from William Shakespeare's masterpiece "*Romeo and Juliet*" clearly demonstrate that a certain plaque was well known in former times and still is tantalizing mankind today: *Herpes simplex virus type 1* (HSV-1). Of course, Mr. Shakespeare was not the first person who was obviously very familiar with the symptoms caused by an acute HSV-1 infection. Already long before the birth of Christ Hippocrates used the term "herpes" from the Greek word meaning "to creep" and provided a detailed description of lesions that could have been caused by HSV-1. Herodotus was the first to associate recurrent fever and the typical lesions of the skin [1]. Although these symptoms and the virus itself have been known for many years, HSV-1 still poses many question and scientific interest for both patients as well as researchers.

42.1.2
The Role of Viral Immediate-early Proteins During the Conquest of the Cell

HSV-1 belongs to the family of large DNA viruses, containing a 152 kb large double-stranded DNA genome. It consists of two pairs of inverted repeats that surround the long (U_L) and the short (U_S) unique sequences. Short repetitive sequences are located at both ends and a total of 89 open reading frames could be identified [2]. The introduction of the DNA microarray technology made it possible to deter-

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mine the exact course of viral gene expression in infected cells. During a replication cycle the genes are expressed in a strictly temporal order and are – according to the time of their expression after infection – divided into immediate-early (IE, α), early (β) and late (γ) gene products [2, 3]. Great attention has been given to the immediate-early (IE) proteins that are of extraordinary importance during the conquest of the cell and the restart of a lytic cycle from latency [4]. The HSV-1 genome encodes for five IE proteins: infected cell protein (ICP) ICP0, ICP4, ICP22, ICP27 and ICP47. The IE genes become activated by the virion associated transactivator VP16, which also functions as a key activator of lytic infection [5]. This activation is controlled via the recognition of a specific sequence motif within the promoters of the IE proteins [6, 7]. Amongst VP16, HSV-1 delivers another key regulatory protein into the cell from the viral tegument (the space between the viral capsid and the envelope) without the need of being initially synthesized within the host: the *virion host shutoff* (vhs) gene. vhs has been characterized as a viral RNase that specifically degrades essential cellular messenger RNAs [8, 9] and has been described as an influence on the surface expression of MHC-class-II molecules [10]. Interestingly, when using a mutant virus strain where the vhs gene was removed, infected immature DC (iDC) retained their ability to become activated by LPS stimulation which is otherwise abolished after infection with a wild-type strain. Furthermore, without expression of vhs the level of the surface marker CD83 strongly increases after addition of LPS, suggesting an important role for vhs during inhibition of DC maturation [11]. Additionally vhs was discussed as (one of) the main mediator(s) of HSV-1 to evade immune responses [12].

The IE- gene product ICP4 functions as a major regulator as it is involved in the expression of early and late viral genes by influencing the basal transcription level [13, 14]. It interacts with the cellular transcription factor TFIID and complexes with another cellular protein named TAF250 [14]. ICP27 is the only IE-protein that is conserved among all herpes viruses and is essential for lytic replication cycle. It has been described as a nuclear–cytoplasmic shuttle protein that facilitates the export of viral messenger RNAs from the nucleus to the cytoplasm [15], especially of transcripts that do not contain introns and are therefore not exported via the standard export pathways. Thus, ICP27 is involved in posttranscriptional regulation of HSV-1 [13, 16]. ICP22 contains two nuclear localization signals [17] and enhances the levels of late gene expression on posttranscriptional level. It has been reported that ICP22 plays an essential role for optimal virus replication in primary human cells, indicating that its activity depends on the type of the infected cell [18].

One IE-gene product of HSV-1 that has been directly associated with immune escape mechanisms is ICP47. This protein is able to block the presentation of antigens by complexing with and thereby inhibiting the transporter associated with antigen presentation (TAP). This leads in consequence to a lack of MHC-class-I peptide-complex assembly [19, 20]. Furthermore, ICP47 has been shown to influence the neurovirulence of HSV-1 by inhibiting the $CD8^+$ T-cell mediated protection [21].

The remaining IE-species, i.e. ICP0, plays a very special role during the viral replication, although this viral gene product is not essential for virus growth and is also not required if cells are infected with a high virus dose [22]. In the absence of

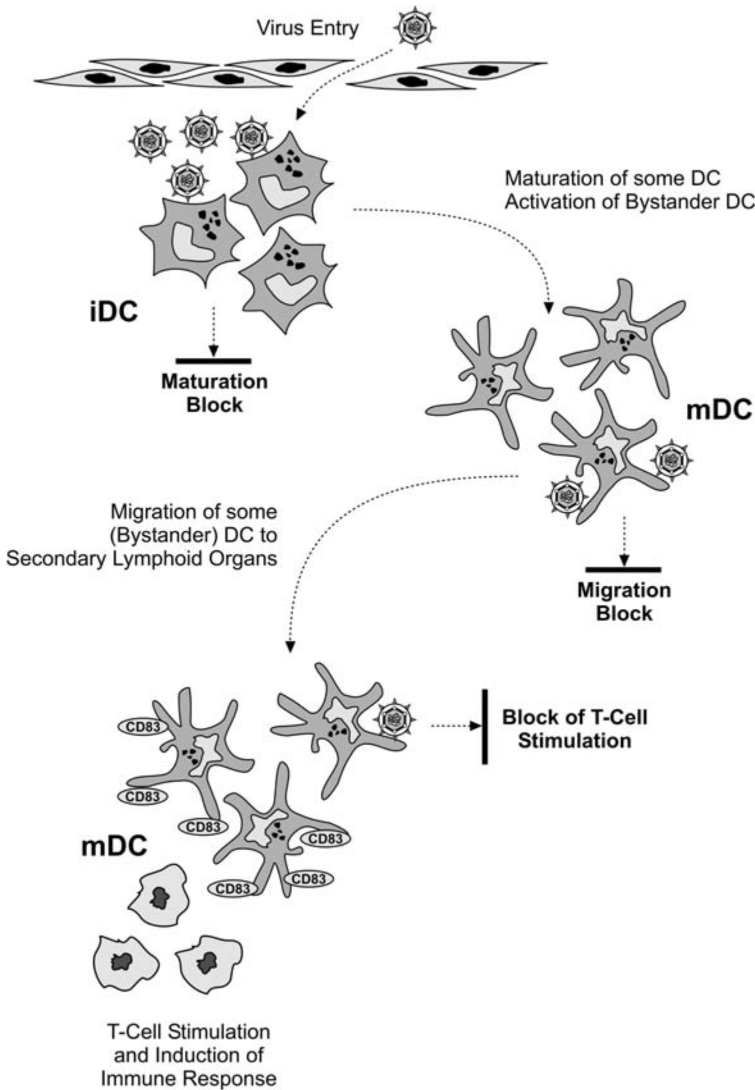

Fig. 42.1 Model for the possible induction/prevention of HSV-1 specific immune responses.

ICP0 a lytic replication cycle is initiated very poorly [23]. Furthermore, ICP0 has been reported to play an important part during the re-activation of quiescent viral genomes and re-activation from latency [24]. Additionally it has been reported, that ICP0 functions as an ubiquitin E3-ligase and thereby marks cellular proteins for rapid degradation via the ubiquitin–proteasome pathway. ICP0 possesses a RING-finger domain that is characteristic of E3-enzymes [25–27]. A well characterized ex-

ample for the influence of ICP0 on the stability of a cellular protein is p53. Boutell and Everett were able to show that ICP0 interacts with and ubiquitinates the major cellular oncoprotein p53. This leads in consequence to a protection against UV-irradiation-induced apoptosis of infected U2OS cells [28]. Finally, it has been shown by Eidson and co-workers that ICP0 inhibits the expression of interferon-stimulated genes and might thereby inhibit cellular antiviral responses [29].

42.1.3
The Course of *Herpes Simplex Virus Type 1* Infection and Replication

Depending on the geographical region HSV-1 can be detected among various populations in 60–95% of all individuals [19, 30]. Many people carry a latent HSV-1 virus in their nervous system without taking notice of its presence. However, they will usually encounter several re-activations of their own private virus during their life.

The infection with HSV-1 can cause a variety of disorders, like primary and recurrent infections of epithelia (e.g. *Herpes labialis* and *Herpes genitalis*), gastro-intestinal complications, corneal infections and retinitis [30–32]. Furthermore, the involvement of HSV-1 in central nervous system disorders has been discussed, as several studies suggested a link between the virus and Alzheimer's disease in adult individuals [33], with meningitis as well as with encephalitis in neonates [34].

Under normal circumstances, the infection with HSV-1 is initiated by the attachment of viral particles to the extracellular matrix of mucosal surfaces or abraded skin. Virions bind through specific interactions with the glycoprotein B and C to heparin sulfate proteoglycans expressed on the cell surface. Following this first essential step, another viral membrane protein i.e. glycoprotein D interacts with cellular receptors, such as the *Herpesvirus* entry mediators Hve-A, Hve-B and Hve-C [35]. This contact is the signal for the fusion of the cellular and the viral membrane. However, the exact mechanisms for the cell entry are still unknown.

The membrane fusion leads to the uncoating of the virus and to the release of nucleocapsids and the tegument proteins, i.e. VP16 and vhs protein, into the cytoplasm. While VP16 is, just like the viral nucleocapsids, transported to the nucleus [36, 37] the vhs protein remains in the cytoplasm and mediates the degradation of cellular mRNAs and the disruption of polysomes [38]. The viral nucleocapsids then liberate the genome directly into the nucleus where VP16 stimulates the transcription of IE-genes. Following the expression of early and late genes in a strict temporal order, the replication of the viral genome and the assembly of the newly synthesized viruses is initiated (for a detailed review of the genome replication: see Lehman and Boehmer [39]).

The viral capsids are assembled in the nucleus, followed by the budding through the nuclear membrane [40]. Afterwards the capsids can be found as complexes with the tegument proteins VP16 and vhs [41]. The readily processed infectious viruses are released by exocytosis in order to infect new cells.

During the acute phase of the infection, HSV-1 has the possibility to enter cutaneous sensory axons and is then retrogradely transported to neuronal cell bodies. There the hallmark of herpesviruses is established, the latency. Latency describes

the ability of the virus to remain in neurons of dorsal root ganglia in an intact and genetically identical form, when compared with infectious viral particles [40, 42]. The HSV genome remains in an extrachromosomal state and latency associated transcripts (LATs) are expressed [40, 43, 44]. The question of how the outcome of a HSV infection (latency or lysogeny) is controlled is subject to many speculations. One idea hypothesizes that during the axonal transport a decrease of the amount of the viral VP16 protein occurs and that this leads to induction of latency [45]. Another hypothesis for the establishment of latency is that it might be due to a lack of appropriate cellular transcription factors (TF) in neurons – TF that usually interact with VP16 and enhance viral gene expression [7, 46]. Finally, it is suspected that the IE-protein ICP0 is involved in regulating the balance between lysogeny and latency [6, 22, 23].

Re-activation of the latent virus is induced by a variety of different stimuli, such as exposure to UV-light, high temperature or immunosuppression. Due to these stimuli the virus emerges from its hiding place and restarts the lytic replication, usually accompanied by the typical herpes lesions [42, 47].

42.2 *Herpes Simplex* Virus meets Dendritic Cells

42.2.1 The Way into Dendritic Cells

42.2.1.1 Receptors and Ligands for Cell Entry

The infection of a cell by a virus is usually a process that consists of many different phases: the first step is the uptake of the virus into the host cell, followed by the expression of viral genes and the replication of the viral genome. Finally the assembly of viral gene products leads to the production of new viral particles.

Thus, the first part of the virus replication cycle depends on several receptors expressed on the cell surface and on several proteins expressed by the virus itself [35]. DC have multiple surface molecules that are exploited or function as receptors for different viruses: for example the human immunodeficiency virus type 1 (HIV-1) uses receptors like CD4, CCR5 or CXCR4, all known to be well expressed on DC [48, 49]. Furthermore, DC-SIGN, another DC specific molecule, has recently been identified as a entry mediator for different viruses, including HIV-1 [50].

The necessary molecules for HSV-1 entry are well expressed by both immature and mature DC: interaction of the viral glycoproteins B (gB) and C (gC) with heparan sulfate proteoglycans arranges the initial attachment to the cell surface [49, 51, 52]. Next the viral glycoprotein D (gD) binds to specific surface receptors, named Hve-A, Hve-B and Hve-C [52]. Hve-A, formerly known as the herpesvirus entry mediator (HVEM) is a member of the TNF/nerve growth factor (NGF) receptor family [49, 53]. Hve-B (formerly known as Prr-2) and Hve-C (formerly known as Prr-1) represent members of the Ig superfamily. Interestingly, the expression of Hve-B is upregulated during DC maturation [51]. The interactions of viral com-

partments with these receptors finally lead to the uptake of the virus into the cell and to the release of the viral genome.

42.2.1.2 Infection of Different Dendritic Cell Populations by *Herpes Simplex Virus Type 1*

The question whether viruses, especially HSV-1, are able to infect certain subpopulations of DC, has been addressed by several groups. Under normal circumstances, on its way to the infection of keratinocytes of the human epidermis via slightly damaged skin, immature DC (iDC; also referred to as Langerhans cells; LC) represent the first group of antigen presenting cells (APC) that HSV-1 will be confronted with [54, 55].

The ability of HSV-1 to infect iDC has been reported by Mikloska and co-workers [56]. They provided evidence that the infection of immature monocyte-derived DC leads to the expression of immediate-early, early and late viral proteins as well as of glycoprotein D. Furthermore the production of moderate amounts of infectious particles has been reported. Interestingly, approx. 12 h after the infection a dramatic loss of surface molecules, including CD1a, CD40, CD54 has been observed. During ongoing viral replication further essential molecules i.e. CD80 and CD86 were downregulated from the cell surface, while in contrast other markers such as CD11c or MHC class I and II were not affected. As HSV-1 infection of iDC leads to significant cytopathic effects (20–45% of iDC die within 24–48 h) [56, 57] the elimination of viable DC and/or the inhibition of DC maturation might represent an efficient way to overcome the induction of naïve antiviral immune responses, a task that can only be carried out by fully matured DC (mDC) [58].

Thus, an additional viral strategy to overcome the induction of potent antiviral immune responses would be the inhibition of mature DC maturation. In this respect, Kruse et al. [59] reported that mDC can be very efficiently infected with HSV-1 at a multiplicity of infection (MOI) of 1. In strong contrast to iDC the infection of mDC does not lead to the generation of infectious viral particles and in consequence, at this particular MOI of 1, does not lead to virus-induced cell death, either. Interestingly, only viral transcripts of immediate-early and early genes were detected [59]. Even more interesting are the phenotypical changes of HSV-1 infected DC: first of all a cluster formation of the cells was observed approx. 10 h post infection. FACS analysis of infected DC revealed a dramatic influence on the cell surface expression of the DC-specific molecule CD83, which was nearly completely downmodulated from the cell surface. CD83 is the best-known cell surface marker for mDC [60] and plays an essential role during the induction of T-cell mediated immunity [61–63]. Influence on other molecules such as CCR7 and CXCR4 – which represent essential markers for chemokine mediated migration of mDC [64–66] – has been reported recently [67] and will be discussed below. Nevertheless, not all molecules, which are expressed by mDC, are influenced by HSV-1 infection. This includes amongst others CD25, CD40, CD80, CD86 as well as MHC molecules [59].

However, since infected individuals are usually able to mount an antiviral immune response, the question is "how" this is induced. Most probably not all iDC

are infected and thus viral antigens from infected DC could be uptaken by uninfected bystander DC that mature, migrate to the lymph nodes and induce an antiviral response via so called crosspriming. The mechanism to induce immune responses by crosspresentation has been reported for several viruses including *Vaccinia virus, Influenza virus, Polio virus,* HIV-1, EBV as well as HCMV [68–71].

Furthermore, Pollara et al., reported data, supporting a pathogenic model of HSV infection, in which initial delay in the generation of immune responses to HSV at peripheral sites is mediated by disruption of DC function but is overcome by bystander DC maturation and crosspresentation of HSV antigens [55]. This observation was fed with further evidence by a recent study from the same group where they demonstrated that infected DC as well as uninfected bystander DC develop into a more mature phenotypical state. Additionally, they were able to demonstrate that myeloid DC, which represent the group of APC that would first get in contact with HSV-1 inside the host, can activate uninfected bystander DC by releasing type I interferon (IFN). The release of type I IFN has also been reported for HSV-1 infected mice by Krug et al. and is mediated via the Toll-like receptor 9 (TLR 9)/MyD88 pathway [72]. However, direct contact of cells with viral particles was necessary for the maturation of these bystander DC by the activation of NF-κB and p38 MAPK pathways and the release of type I IFN [52]. Furthermore, Bosnjak et al. were able to demonstrate that T-cell activation in response to a HSV-1 infection is mediated by nonepidermal subsets of DC, again through the mechanism of crosspresentation. This study demonstrates that HSV-1 infected apoptotic DC will be phagocytosed by uninfected DC and are subsequently able to stimulate specific antiviral immune responses [73]. Finally, in a naturally occurring HSV-1 infection the balance between activation of DC maturation and inhibition of DC function will probably determine whether or not an immune response can be induced [55].

42.2.2 Interference with Typical Functions of Dendritic Cells

42.2.2.1 Interference with Dendritic Cell Maturation

Immature DC are usually the first APC with which HSV-1 will get in contact during the infection of the host. These iDC reside in the periphery and are specialized sentinels to take up and process antigens, but unlike mDC, they are unable to induce potent immune responses and are rather involved in the induction/maintenance of tolerance mechanisms [58]. Maturation of iDC is induced by direct contact with various products of infectious agents or via inflammatory stimuli [74–76]. Thus, the inhibition of maturation right after the infection by either inducing apoptosis or by blocking the surface expression of essential molecules on both iDC and mDC will definitely decrease DC-mediated T-cell activation.

The specific inhibition of DC maturation by HSV-1 was first described by Salio and co-workers [51]. They infected iDC with a recombinant disabled infectious single cycle (DISC) HSV-1 and demonstrated that infected iDC are no longer able to upregulate co-stimulatory molecules that are essential for activation of DC. Even the addition of LPS to the infected DC did not induce maturation. In addition the

infection led to a lack of cytokine secretion such as interleukine (IL)-6, IL-10 and IL-12. Interestingly, infected iDC also failed to upregulate the surface molecule CCR7 which is an essential mediator of chemokine induced migration towards secondary lymphoid organs, and furthermore represents an essential marker for mDC. This observation has recently been confirmed and further investigated with a fully replication competent wild-type strain of HSV-1 [67]. A particular candidate of viral proteins that might cause the failure of DC activation has been identified by Samady and co-workers: the virion host shutoff gene (vhs). By using a virus mutant strain missing the vhs gene, they demonstrated that DC infected with this particular strain can be activated by LPS after infection [11].

Another efficient way to inhibit maturation of iDC would be by the induction of cell death. The induction of apoptosis by HSV-1 has been reported recently by Müller et al. [57]. They demonstrated that following HSV-1 infection of iDC the caspase-8-dependent apoptosis was strongly induced. This was the consequence of a viral modulation of apoptosis regulating proteins in iDC. The induction of apoptosis is controlled by several cellular proteins including an inhibitor of the procaspase-8 (FLICE) named cellular FLICE inhibitory protein (cFLIP). This protein exists in two splice variant (a short = $cFLIP_S$ and a long = $cFLIP_L$) that usually control the recruitment of FLICE into the death inducing signalling complex (DISC) and thereby inhibits apoptosis. Following infection with HSV-1, the expression of $cFLIP_L$ was strongly reduced and the expression of the apoptosis inducing protein p53 was upregulated. This led to the virally induced apoptosis of the major part of the infected cells. In this connection it is very interesting to know that only iDC were sent to death upon infection, while infected mDC were not [59].

It is noteworthy, that the inhibition of DC maturation is not a single feature of HSV-1, but has been reported for several other viruses including *Herpes simplex virus type 2* (HSV-2) [77] and *Vaccinia virus* [78]. Furthermore, the matrix protein M1 of *Influenza virus* reduces the ability of infected DC to stimulate cytotoxic T lymphocytes [79] and the *Human cytomegalovirus* (HCMV) interferes amongst others with the upregulation of MHC molecules [80]. Recently it has been reported that iDC, which were inoculated with the HIV-1 specific protein Vpr completely failed to mature and to develop into mDC [81]. Thus the influence on DC maturation is a widespread mechanism among several viruses to overcome the human immune system.

42.2.2.2 **Interference with Dendritic Cell Migration**

As described above, HSV-1 infected iDC are unable to mature, however they are able to activate bystander DC via the release of type I IFN and the mechanism of crosspresentation. In this respect the host has developed a strategy to overcome this viral-immune escape mechanism. However, viruses would not have been that successful over the centuries, without developing additional ways to survive within their hosts.

After the uptake and processing of antigens, the maturing DC have to leave the periphery and migrate into the T-cell areas of the secondary lymphoid organs [58, 82–85]. Therefore, maturing DC undergo a rapid and strictly regulated switch

(-known as the chemokine receptor switch) in the expression of certain chemokine receptors [83, 86]. While iDC respond to many CC- and CXC-type-chemokines such as CCL3, CCL4 and CCL5 to find their way into inflamed tissues, the expression of the corresponding receptors is reduced or completely abolished in mDC [83, 86–88]. In strong contrast to iDC, mDC show a strongly increased responsiveness to CCL19 and CXCL12, two chemokines that are expressed in high levels by secondary lymphoid organs [64, 88–90]. Indeed, the lack of the chemokine receptor CCR7 led to defects in lymphocyte homing and DC location [91], demonstrating the absolute essential role of this receptor for the induction of primary immune response [90, 92]. Viral influence on chemokine receptor expression would therefore be another way to escape the host's surveillance system.

Indeed, an influence of HSV-1 on the expression of migration mediating molecules has been described both for iDC and mDC. Salio and co-workers infected both types of DC with a DISC-HSV-1-GFP virus mutant, capable of completing only one single cycle of infection. Using this type of virus, they could show that infected iDC did not acquire responsiveness to CCL19 during maturation with LPS, while infected mDC were not affected by the virus in their experiments [51]. Evidence that mDC might also be affected in their migration capability after the encounter with HSV-1 was provided Toka and co-workers when they over-expressed amongst others the chemokine CCL19 [93]. This led to a very efficient enhancement of a protective immune response against HSV-1. The CCR7 ligand (CCL19) functioned as a molecular adjuvant, improving the host's response towards the invading pathogen [93]. It could be possible that the increased expression of CCL19 simultaneously increased the migration of immune competent DC. Furthermore, Okada et al. demonstrated the importance of the CCR7/CCL19-mediated migration using an adenoviral vector to transduce DC with the CCR7-expressing gene. The transduced DC expressed high levels of CCR7 which correlated with a significant augmentation of the migratory capacity [94]. Finally, Eo and co-workers reported that the co-delivery of a CCR7 ligand expressing DNA is a useful adjuvant for the vaccination against HSV-1 [95] and that functional defects of $CD8^+$ T-cells could be corrected by the use of DNAs that expressed the CCR7 ligands [96].

The *Human Cytomegalovirus* (HCMV) represents another member of the *Herpesviridae* family, and in this respect an influence on both iDC as well as mDC migration has been described. The infection of iDC with HCMV inhibited the migration of these cells by downregulation of the surface receptors CCR1 and CCR5 [97]. These two chemokine receptors usually mediate the migration of blood-DC precursors and iDC towards tissues expressing chemotactic stimuli as a consequence of inflammatory stimuly. Moutaftsi et al. reported that DC which were infected in their immature state with HCMV and received maturation signals afterwards still showed ability to undergo the first step in chemokine receptor switching, i.e. the downregulation of CCR5. However, they were unable to complete the next step essential for initiation of migration, i.e. the upregulation of CCR7 [98].

Also in case of HSV-1 a powerful effect on chemokine mediated migration has been described: when mDC were infected with the virus at a MOI of 1 a significant loss of mRNA encoding the chemokine receptors CCR7 and CXCR4 was detectable

[67]. As a consequence, CCR7 and CXCR4 expression was also abrogated at the protein level, resulting in a strong reduction of chemokine-mediated migration. In addition it has been shown that the observed downregulation is not a consequence of a general degradation of cellular mRNAs by the vhs [67]. However, the responsible viral gene product(s) still remains to be identified.

Thus, influencing DC-migration represents another powerful viral mechanism to block the induction of potent antiviral immune responses.

42.2.2.3 Interference with Dendritic-cell-mediated T-cell Stimulation

Finally, the induction of strong T-cell responses represents a very critical step during the induction of antiviral immune responses by DC. To perform this important task, mDC have to migrate to the draining lymph nodes, were naïve T-cells interact with mDC.

Smith et al. reported, that in mice, which were infected with HSV-1 via the footpads, only CD8-α^+ DC were involved in the mediation of CTL immune responses against the virus [99]. Furthermore, van Lint and co-workers were able to demonstrate that $CD8^+$ T cells were able to clear established lytic infections from skin and nerves. In addition, this T-cell subpopulation was partially responsible for the limitation of virus spread within the skin [100]. In a different study, Stock et al. reported that a prolonged antigen presentation was detectable after HSV-1 skin infection in mice. Usually antigen presentation was detectable within hours after the infection with viruses, bacteria or parasites, but was abolished almost completely within two days, probably by the lysis of APC, within a feedback mechanism. Surprisingly, the period of antigen presentation and the priming of naïve $CD8^+$ T cells was prolonged up to seven days by the infection with HSV-1 [101].

Further evidence regarding the influence of HSV-1 on DC-mediated T-cell activation came from Björck et al. [102]. When the authors exposed plasmacytoid DC (pDC) to HSV-1 *in vivo*, they produced the usual large amounts of IFN-α. However, when these DC came in contact with HSV-1 again *in vitro*, the production of IFN-α was almost completely abolished. Furthermore, a strongly reduced T-cell alloreactivity was also observed [102].

In order to induce potent antiviral T-cell immune responses via the presentation of MHC–peptide complexes on APC, the viral proteins have to be degraded and loaded onto MHC molecules. This represents another potential step where the virus could interfere with the immune system of the host. Indeed, it has been reported that the viral IE–protein ICP47 blocks the transporter associated with antigen presentation (TAP), thus in HSV-1 infected cells peptides from processed viral antigens cannot reach the area of MHC class I assembly, the endoplasmic reticulum [21], and thus the induction of antiviral immune responses is hampered. Furthermore, apart from $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells also play a very important role in the induction of antiviral immune responses and are thus subject to viral immune evasion mechanisms. In this respect, Trgovcich et al. reported that HSV-1 influences (inhibits) MHC class II antigen presentation via two viral gene products, the vhs protein and the infected cell protein 34.5 [10].

Similar observations were made when human iDC were infected with HSV-1 *in vitro*: the infected cells showed a significant downmodulation of surface molecules which are mandatory for proper immune response initiation [56] and were only poorly able to stimulate T cells [51].

In this respect a very interesting finding was reported by Kruse and co-workers [59]. When mDC were infected with HSV-1 a strong downregulation of the cell surface expression of CD83 was observed [59]. This surface molecule is the best known marker for mDC, is usually specifically upregulated during DC maturation and plays an essential role during the induction of DC-mediated immune responses [61]. As described above, CD83 was almost completely removed from surface by 10 h post infection with HSV-1, while other cell surface molecules including CD80 and CD86 were not influenced. Interestingly, this CD83 downmodulation on mDC, correlated with a reduced T-cell stimulatory capacity. In addition, further experiments demonstrated that CD83 was not just internalized but was completely degraded after the infection with HSV-1, supporting the important role of CD83 [59].

For another member of the *Herpesviridae* family, HCMV, similar effects have been reported. First off all HCMV is able to infect mDC and to inhibit stimulation of T-cell proliferation [80, 103]. It is of particular interest that, as a consequence of the HCMV infection, a soluble CD83-form is shed from the cell surface which subsequently blocks T-cell stimulation [103] just as described using a recombinantly expressed soluble isoform of CD83 [61]. Furthermore, Arrode and co-workers reported a downregulation of CD83 from DC that were co-cultured with HCMV infected fibroblasts, suggesting that a soluble factor(s) is responsible for the mediation of this effect, and indeed, they could demonstrate the release of the soluble transferring growth factor-β1 (TGF-β1) [104].

Taken together, these data indicate that influencing CD83 expression and/or CD83 function, represents a new and powerful viral immune escape mechanism. Thus, next we will focus in more detail on the biological importance of CD83 for DC biology.

42.3 The Cell Surface Molecule CD83

42.3.1 Characteristics of CD83

The glycoprotein CD83 is well known as the most important cell surface marker for mature human DC [58]. During DC maturation, CD83 is strongly upregulated in combination with the co-stimulatory molecules CD80 and CD86 [60]. Recently it has been demonstrated that CD83 can be found as a pre-form inside monocytes, macrophages and iDC, but is only stably expressed on mDC [105] and some activated T cells and B cells [106]. Interestingly, CD83-homologs could also be detected in mice, elasmobranch and teleost fish [107].

Up to date, *in vivo* two different isoforms of CD83 have been reported: a membrane-bound form (mCD83) [60, 108] and a soluble form (sCD83) [109, 110]. The latter is most probably generated by the proteolytic cleavage of the membrane bound CD83-isoform, however the precise mechanism is unknown [109]. Interestingly, increasing concentrations of this soluble CD83 isoform have been detected in a number of patients with hematological malignancies, including in patients with chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) [110]. These data indicate that the soluble CD83 isoform might play a role in the downmodulation of antiproliferative immune responses in these patients.

Noteworthy in this respect is the fact that, as already stated above, several viruses influence the CD83 expression and its function. The infection of iDC with HSV-1 for instance prevented the upregulation of mCD83 [51] and the infection of mDC led to the downregulation and degradation of mCD83 [59]. In both cases, the loss of mCD83 correlated with an impaired T-cell stimulation.

Furthermore, Senechal and co-workers reported that a soluble form of CD83 is shed from the cell surface of HCMV-infected mDC, which subsequently led to a block of T-cell stimulation [103].

42.3.2
Modulation of Dendritic Cell Function by Interference with CD83 mRNA Processing

It has been observed that the expression of CD83 correlates with the expression of the eukaryotic initiation factor 5A (eIF-5A) [111], which has been reported to be involved in the export of cellular mRNAs from the nucleus into the cytoplasm [112, 113]. eIF-5A therefore interacts with the general export receptor CRM1 by binding to the nucleoplasmic part of the nuclear pore complex (NPC). Afterwards it is translocated from the nucleus to the cytoplasm [114]. For its biological function, eIF-5A has to undergo a posttranscriptional processing: two cellular enzymes, the deoxyhypusine synthase and the deoxyhypusine hydroxylase catalyze the spermidine-dependent mechanism of hypusine modification. It is noteworthy that eIF-5A is the only known cellular protein that contains the unusual amino acid hypusine (N^{-}-(4-amino-2-hydoxybutyl lysine) [115, 116]. The expression level of eIF-5A is very low in iDC but increases during DC maturation [111]. When one of the catalyzing enzymes, and in consequence the hypusine modification of eIF-5A, is inhibited by the low molecular weight inhibitor GC7 (N^1-guanyl-1,7-diaminoheptane) DC fail to upregulate CD83 during DC maturation. This is due to a block of the CD83 mRNA export from nucleus into the cytoplasm and goes hand-in-hand with the loss to stimulate T cells [111].

A similar influence on DC activation *in vitro* and *in vivo* has been described by Zinser and co-workers [117]. The addition of CNI-1493 (an inhibitor of the deoxyhypusine synthase) reduced the expression of CD83 during maturation and DC-mediated T-cell stimulation. Furthermore, when applied in an early therapeutic setting it also reduced the clinical symptoms using an experimental autoimmune encephalomyelitis (EAE) model [117].

Furthermore, a viral influence on CD83 mRNA export might also be conceivable, especially when taking in consideration the fact that HIV-1 exploits the eIF-5A transport pathway for efficient export of its own mRNAs [118].

42.3.3 The Soluble Extracellular Domain of CD83 and its Influence on T-cell Proliferation

As reported above, a soluble form of CD83 (sCD83) exists also in the sera of healthy individuals and is released from activated DC and B lymphocytes [109]. Furthermore, in a number of haematological malignancies it can be detected in highly elevated levels [110]. Interestingly, the inhibition of the *de novo* protein synthesis did not influence the release of soluble CD83, suggesting that sCD83 represents a shedded version of membrane-bound form [109].

To investigate whether sCD83 does indeed have important functional properties regarding the DC-mediated T-cell stimulation the extracellular domain of human CD83 (hCD83ext) was expressed as a recombinant protein in *E. coli* and used for functional studies [63, 119]. And indeed, the inoculation of DC with this soluble CD83-isoform or a CD83-Ig fusion protein had several dramatic consequences. First of all DC incubated with sCD83 revealed dramatic changes in the organization of their cytoskeleton. The DC rounded off, had only short or no veils at all and as a direct effect completely failed to form clusters with T cells [120].

In addition, the biological function of these sCD83-treated DC were analyzed *in vitro* using MLR assays. Interestingly, sCD83 inhibited the DC-mediated allogenic T-cell stimulation in a dose-dependent manner [63]. Next, the effect of sCD83 was analyzed *in vivo* using the murine experimental autoimmune encephalomyelitis (EAE) model. Interestingly, the sCD83 was very effective in a prophylactic as well as in a therapeutic application, underlining its high immunosuppressive potential also *in vivo* [121].

The immunosuppressive activity of soluble CD83-isoforms during the development of cellular immunity has also been reported by Scholler and co-workers [122]. The authors implanted immunogenic P815 tumor cells directly into recipient mice and afterwards injected a CD83-Ig-fusion protein intraperitoneally and observed the tumor growth. Interestingly, CD83-Ig-fusion protein treated animals developed twice as large as tumors when compared with untreated mice, suggesting that soluble CD83 downmodulates antitumoral immune responses [61, 122].

Taken together, these results underline the impressive immunomodulatory function of sCD83 both *in vivo* and *in vitro*.

42.3.4 The Function of Membrane-bound CD83

As described above, the main function of soluble CD83 forms is to interfere with DC-mediated immune responses, thus acting as an immunosuppressive molecule. This is in strong contrast to the membrane-bound form of CD83, which definitely has a different function.

To further investigate the functional differences between the sCD83 and the mCD83, Scholler and co-workers constructed fusion proteins of the extracellular domain human CD83, fused to an Ig-domain (i.e. CD83-Ig) [122]. This protein was then co-immobilized with anti-CD3 mAb, thereby simulating a membrane-bound form of CD83. Interestingly, only this co-immobilized protein induced strong proliferation of PBMC, while CD83-Ig alone failed to do so. Removal of adherent cells from the PBMC population led to a 3-fold decrease of proliferation. These results prompted the authors to speculate that the presence of adherent cells determines the activity of immobilized CD83. Furthermore, they were able to show that following co-immobilization of CD83-Ig with anti-CD3 mAB, the ratio of $CD8^+$ to $CD4^+$ T cells increased by a factor of 2.5, suggesting a specific function of CD83 in the induction of $CD8^+$ T lymphocytes [122, 123]. In additional experiments the poorly immunogenic melanoma cell line K1735 was transfected with CD83 and subcutaneously implanted into mice. After one month, one half of the mice were implanted, the other half were injected with K1735 wild-type cells. Interestingly, pre-treated animals demonstrated strongly reduced tumor development. Thus, in this experiment mCD83 seems to strengthen immune responses against tumors [122]. This observation was essentially confirmed by Yang and co-workers, identifying CD137 as a cofactor for increased antitumor immunity of CD83 expressing cells [124].

These data clearly indicate the importance of CD83 for the induction of cellular immune responses and that the manipulation of CD83, as reported for HSV-1, HCMC and other viruses leads to the downmodulation of antiviral immune responses.

42.3.5
Influence of CD83 on the T-cell Development in the Thymus

However, CD83 not only plays an important role in DC biology, but also in T-cell development in the thymus, as recently reported by Fujimoto et al. using CD83-deficient ($CD83^{-/-}$) k.o. mice [125]. Interestingly, these animals showed a profound reduction in their $CD4^+$ single-positive thymocyte development (reduction by 68% compared to wild-type mice) [125]. Furthermore, the $CD83^{-/-}$ mice revealed a 75–90% decrease of peripheral $CD4^+$ T cells. The number of $CD8^+$ single-positive thymocytes was neither reduced nor increased, but in contrast to this, the number of $CD8^+$ T cells in the peripheral blood was strongly increased.

Furthermore, the observations that resting B cells derived from CD83-k.o. mice revealed low MHC class II antigen expression and reduced MHC class II and CD86 production after activation, suggested the necessity for CD83 expression also in respect to the B-cell activation.

Knowing the fact, that $CD4^+$/$CD25^+$ T cells regulate primary and memory $CD8^+$ T-cell responses against HSV-1 [126], interference with $CD4^+$ T-cell development, for instance by influencing the CD83 expression and/or shedding of soluble CD83 isoforms, could be an additional way to influence antiviral immune responses.

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Abbreviations

APC	antigen-presenting cells
DC	dendritic cells
eIF-5A	eucaryotic initiation factor 5A
gB, gC, gD	glycoprotein B, C, D
HCMV	human cytomegalovirus
HSV-1	*Herpes simplex virus type 1*
Hve	herpesvirus entry mediator
ICP	infected cell protein
iDC	immature dendritic cells
IE	immediate early (protein)
IFN	interferon
LPS	lipopolysaccharide
MOI	multiplicity of infection
mRNA	messenger RNA (ribonucleic acid)
mDC	mature dendritic cells
NPC	nuclear pore complex
TF	transcription factors

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43
Epstein–Barr Virus

Christian Münz

43.1
The Epstein–Barr Virus (EBV)

The Epstein–Barr virus (EBV) is a human γ-herpesvirus, which infects more than 90% of the human adult population [1]. After infection, EBV persists life-long, primarily colonizing the B-cell compartment of its host [2]. During persistence the two principal EBV infection programs, latent and lytic, are found in different B-cell differentiation stages of healthy EBV carriers. Latent infection with the expression of all latent EBV antigens (six nuclear antigens: EBNA1, 2, 3A-C and LP, and two membrane proteins: LMP1 and 2) can be found in naïve tonsillar B cells. Germinal center B cells express only EBNA1, LMP1 and 2. EBV latent antigen expression is further downregulated in peripheral blood memory B cells, which in their majority express no EBV latent antigens. The lytic EBV infection with more than 60 viral products [1] can be activated from this memory B-cell pool, presumably after encounter of the cognate B-cell receptor antigen by the respective EBV infected memory B cell. In healthy virus carriers, lytic EBV infection can be found in tonsillar plasma cells [3], which shed virus into the saliva for further transmission to other humans. Therefore, EBV infection in healthy virus carriers is confined to distinct antigen expression in different stages of B-cell development.

Despite its asymptomatic, but life-long, infection in most adults, EBV causes transformation of B cells *in vitro* and tumors of B-cell, epithelial as well as more rarely T-cell and natural killer (NK)-cell origin *in vivo* [4]. Similar to the plasticity of latent EBV infection in healthy EBV carriers, different EBV-associated malignancies express all or only subsets of latent EBV antigens. B-cell tumors carrying all latent EBV antigens like lymphoproliferative disease are only found in immune suppressed individuals. Of the spontaneously arising EBV-associated malignancies, Hodgkin's disease mirrors latent EBV antigen expression in germinal center B cells, carrying EBNA1, LMP1 and LMP2 [5, 6], while other prominent EBV-associated tumors like nasopharyngeal carcinoma and Burkitt's lymphoma have even further restricted latent EBV antigen expression patterns [4]. The only EBV protein expressed in all EBV-associated malignancies is EBNA1. Therefore, EBV-associat-

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ed malignancies display latent EBV antigen expression patterns similar to those found in B cells of healthy virus carriers and control of these latency programs seems essential to prevent tumorigenesis. In light of the high penetration that EBV achieves in the human population with more than 90% of adults carrying persistent infection, EBV is one of the essential pathogens humans need to resist for their survival and the human immune system is crucial for the control of EBV.

In this chapter, I will discuss the immune responses that control latent EBV infection and the role that dendritic cells (DCs) play in the activation and priming of immunity against EBV. Furthermore, I will draw parallels to other herpesviruses and speculate how DCs might detect EBV infection and close by arguing for the use of DCs in immunotherapy of EBV-associated malignancies.

43.2
Immune Control of Epstein–Barr Virus

Apart from oral hairy leukoplakia, which is the result of uncontrolled lytic EBV replication on the tongue, all EBV-associated diseases are caused by latent EBV infection. Especially, latent EBV antigens drive cellular transformation, and immune control of EBV latency, therefore, becomes crucial in the resistance to tumorigenesis in healthy EBV carriers, namely the vast majority of the human adult population. Persistent immune control in the face of life-long EBV infection rests mainly on the shoulders of the T-cell immune system. This becomes apparent in individuals after therapeutical immunosuppression after transplantation [7], hereditary immunosuppressive disorders like X-linked lymphoproliferative disease [8, 9] and immunosuppression by coinfection with HIV [10]. In all these conditions EBV-associated malignancies develop at increased frequencies. Post-transplant lymphoproliferative disease can be efficiently cured by adoptive transfer of EBV-specific T cells [11, 12], indicating that lack of EBV-specific T-cell immunity results in tumorigenesis in these patients. It has been proposed that, in addition to T cells, NK cells are essential in the immune control of EBV-transformed B cells. *In vivo* they were required for the prevention of EBV-associated lymphoproliferative disease in PBMC transplanted SCID mice [13]. The lack of EBV-transformed B-cell recognition by NK cells contributes to the fatal outcome of primary EBV infection in patients with X-linked lymphoproliferative disease [14]. Furthermore, *in vitro* NK cells were found to inhibit B-cell transformation by EBV [15–17]. T cells and NK cells share cytolytic capacity as well as the ability to secrete the antiviral cytokine IFNγ and both seem to play a role in the immune control of EBV.

The activity of NK cells is regulated by two groups of receptors, activating and inhibitory NK receptors. The main activating NK-cell receptors are the natural cytotoxicity receptors (NCRs) and NKG2D [18]. In the only study addressing NK-cell recognition of EBV-transformed B cells to date, NK cells were found to detect EBV-transformed B cells either exclusively via NCRs or by a combination of NCRs and NKG2D [19]. While the ligands for NCRs on EBV-transformed B cells are unknown, two EBV-transformed B-cell lines were found to express the NKG2D li-

gands ULBP 1 and 3 (UL16 binding proteins 1 and 3). All NKG2D ligands are stress-induced molecules, which alarm the immune system in response to cellular transformation and viral infection. On the contrary, the inhibitory receptors of the KIR and the CD94/NKG2 families engage classical and nonclassical MHC class I molecules and dampen the recognition of autologous EBV-transformed B cells by NK cells. These findings suggest that NK cells target EBV-transformed B cells via their NCR receptors and in some instances NKG2D receptor recognition might contribute to NK-cell-mediated resistance to B-cell transformation by EBV.

NK-cell responses to EBV-transformed B cells might limit EBV-induced B-cell proliferation initially prior to the onset of protective T-cell mediated immune control. In addition, NK cells might continue contributing to the immune control against EBV-associated malignancies with low immunogenicity for T-cell responses like Burkitt's lymphoma [20, 21]. The general paradigm in EBV immunobiology, however, is that T cells shoulder the main load of EBV immune control. Interestingly, $CD4^+$ and $CD8^+$ T cells thereby mount a comprehensive immune control targeting the whole spectrum of EBV latent antigens with distinct antigens being preferentially recognized by $CD4^+$ or $CD8^+$ T cells [22]. Namely the EBNA3 antigens are dominant $CD8^+$ T-cell antigens and subdominant responses have been detected against EBNA1, LMP1 and 2 in healthy EBV carriers [23–30], while $CD4^+$ T cells consistently recognize EBNA1 with subdominant responses to EBNA3C and LMP1 [31–35]. Interestingly, both $CD4^+$ and $CD8^+$ T cells recognize EBV-transformed B cells [36–38] and seem capable to prevent B-cell transformation by EBV [26, 39–42]. IFNγ secretion by latent EBV antigen-specific T cells seems sufficient to prevent B-cell transformation [26, 43] and it remains unclear to which extend cytolytic effector cells are required for EBV-specific immune control. These studies suggest that all EBV latent antigens are recognized by either $CD4^+$ or $CD8^+$ T cells and that therefore all EBV latency programs, even the EBNA1 only program of Burkitt's lymphoma are under the immune control by the human immune system.

The initiation of this comprehensive immune control of EBV by NK cells and T cells is of particular interest, because it prevents tumorigenesis in nearly everybody, since most adults are infected by EBV. Understanding the establishment of such a tight immune control might also enable us to target other human malignancies via the initiation of a similar comprehensive antitumor immune control.

43.3 Stimulation of Lymphocyte Compartments Relevant to Epstein–Barr Virus Immune Control *in vitro*

43.3.1 Tonsillar Natural Killer Cell Activation by Dendritic Cells and its Possible Role in Epstein–Barr Virus Infection

EBV enters the human body via the tonsils after saliva exchange, and successful immune control of the virus in asymptomatic seroconverters seems to be entirely

handled in the tonsils [44]. Therefore, the lymphocyte content of human tonsils should be crucial for immunity to EBV. Human tonsils contain a distinct subset of NK cells, which are most likely precursors of the cytolytic NK cells in peripheral blood, but rapidly secrete cytokines upon activation [45]. With respect to EBV infection tonsillar NK cells are potent secretors of IFNγ, which has been shown to prevent B-cell transformation by EBV. The tonsillar NK cells are similar to the minor NK-cell subset in peripheral blood and these so-called $CD56^{bright}$ NK cells have been described to secrete higher amounts of IFNγ, GM-CSF and TNF than the major NK-cell subset in human peripheral blood, the classical cytolytic $CD56^{dim}$ NK cells [46, 47]. The $CD56^{bright}$ NK cells from peripheral blood carry homing markers for secondary lymphoid tissues [48] and are indeed enriched in lymph nodes and spleen in addition to tonsils [45, 49]. Therefore, the unique NK-cell population in tonsils could play a crucial role in EBV-specific immune control via IFNγ secretion.

The physiological activation of NK-cell responses is probably mediated by DCs. Both in mouse and man, DCs can activate NK cells to proliferate, secrete IFNγ and increase their cytolytic capacity [50–53]. While human NK activation by DCs was initially investigated with peripheral blood NK cells, recently it was also demonstrated for DCs and NK cells from human secondary lymphoid organs [54]. DCs and NK cells indeed also co-localized in the parafollicular T-cell areas of human lymph nodes [49, 54]. In addition, DCs were found to selectively activate $CD56^{bright}$ NK cells [54, 55], the NK-cell subset enriched in human secondary lymphoid organs. NK-cell activation by DCs was found to be critically dependent on IL-12 and IL-15 production by DCs in both mice and man [54–58]. Based on these studies, others and we suggested that human secondary lymphoid tissues are the main interaction site for DCs and NK cells [59, 60].

Apart from controlling B-cell transformation by IFNγ in tonsils, NK cells in secondary lymphoid organs might influence the efficiency and polarization of EBV-specific T-cell priming. In mice DCs have indeed been shown to call NK cells into lymph nodes, which then polarize primed T-cell responses towards Th1 [61]. Similarly in humans, NK cells have been shown to influence DC maturation to more efficiently induce Th1 immunity [62]. Therefore, NK cells in tonsils might not only fight B-cell transformation by their superior cytokine secretion ability, but also polarize the emerging EBV-specific immune response towards an antiviral Th1 phenotype. DCs seem to be crucial to activate NK cells in secondary lymphoid organs for these effector functions.

43.3.2
Initiation of Epstein–Barr Virus-specific T-cell Immunity by Dendritic Cells

Probably with the support of NK cells efficient T-cell immunity against EBV is primed. Since EBV infects B cells and B cells are considered antigen presenting cells (APC), which can possibly initiate immune responses by themselves, the question arises if EBV-transformed B cells initiate EBV-specific T-cell immune control or require a bystander APC to achieve this task [63]. Studies *in vitro* had demonstrated that while EBV infection of peripheral blood mononuclear cells

(PBMCs) from EBV seropositive donors did not lead to outgrowth of EBV positive B-cell lymphomas and EBV-transformed B cells regressed with fast kinetics, regression of EBV-transformed B cells did not occur in EBV infected PBMC cultures of EBV seronegative individuals [64]. These findings already indicate that EBV infected B cells are unable to prime protective T-cell immunity *in vitro*. More recently we found that addition of DCs induces regression of EBV-transformed B cells in EBV seronegative cultures [42]. In this study we could show that T-cell responses are only primed in the presence of DCs in EBV seronegative donors and that inhibition of T-cell reactivity with cyclosporin A reversed regression. Moreover, regression of EBV-transformed B cells in EBV seronegative cultures in the presence of DCs occurred with delayed kinetics compared to EBV seropositive cultures. With regression occurring between day 11 to 14 after infection, the kinetics resembled an primary immune response in EBV seronegative cultures, while with regression already detectable at day 3, EBV seropositive cultures showed the kinetics of an secondary immune response. In our hands, both $CD4^+$ and $CD8^+$ T cells were able to mediate regression in these cultures. These data indicated that DCs are the initiators of protective T-cell immune control against EBV *in vitro*.

EBV does not seem to establish latent infection in DCs [42, 65], but latent EBV antigen-specific T cells are probably crucial for regression in EBV infected cultures. Myeloid DCs might, however, be susceptible for virus entry without establishment of productive infection [66]. Instead of direct infection of DCs by EBV DCs can expand and prime EBV-specific T cells after crosspresentation of fragments from EBV-transformed B cells [42, 67–71]. EBV-transformed B cells in contrast could only prime EBV-specific T cells from EBV seronegative donors when the cultures were supplemented with the DC derived cytokine IL-12 [72] or when $CD25^+$ T cells were selected during stimulation [69]. When peptide pulsed DCs and EBV-transformed B cells were compared side-by-side for stimulation of EBV-specific $CD8^+$ T cells, DCs were always superior in the expansion of cytolytic as well as cytokine secreting $CD8^+$ T cells against latent and lytic EBV antigens [73, 74]. These studies suggest that DCs prime EBV-specific T-cell immune control after cross-presentation of EBV antigens from EBV-transformed B cells.

43.4 Evidence for Priming of Epstein–Barr Virus Immune Control by Dendritic Cells *in vivo*

43.4.1 Strong Th1 Polarization of $CD4^+$ T-cell Responses to the Nuclear Antigen 1 of Epstein–Barr Virus (EBNA1)

Th1 immune responses are orchestrated by IFNγ secreting $CD4^+$ T cells and are thought to mediate their superior antiviral and antitumor effects mainly by IFNγ secretion and cytolytic activity with $CD8^+$ T cells as the final effectors of this cell-mediated immunity [75–77]. Induction of Th1 immune responses seems to be at

least in part dependent on IL-12 [78] and might be supported by IFNγ secreting NK cells [61]. Both IL-12 production and NK-cell activation are exquisitely performed by DCs and DCs are also considered to be superior inducers of Th1 immunity [79]. Therefore, DCs are probably responsible for the priming of strongly Th1-polarized immune responses.

A strong Th1 polarization was found for $CD4^+$ T-cell responses against the nuclear antigen 1 of EBV (EBNA1) [80]. The EBNA1-specific $CD4^+$ T-cell response was dominated by IFNγ secretion in PBMCs of healthy EBV carriers. Moreover, only IFNγ secreting EBNA1-specific $CD4^+$ T cells displayed cytolysis against EBNA1 expressing targets, including EBV-transformed B cells. Since EBNA1 is also a dominant antibody antigen in healthy EBV carriers [81], distribution of antibody isotypes in the humoral EBNA1-specific immune response could be used to evaluate the $CD4^+$ T-cell polarization that assisted in antibody production. It was found that EBNA1-specific antibody responses are dominated by IgG1 [80], an isotype, which is associated with Th1 responses in humans [82]. Therefore, the strong Th1 polarization of EBNA1-specific $CD4^+$ T-cell responses implicate DCs in their priming *in vivo*.

The Th1 polarization of EBV-specific immune control seems to be particularly crucial, because patients with EBV-associated malignancies display signs of Th2 polarization [63]. In Hodgkin's lymphoma for example, which is associated with EBV in 40–60% of all cases, the tumor microenvironment as well as the systemic cytokine profile reveal elevated levels of IL-6, IL-10, IL-13 and TGFβ [83–86]. High levels of these cytokines correlate with poor prognosis for these patients. Furthermore, Hodgkin Reed-Sternberg, the EBV-transformed tumor cells in Hodgkin's disease, secrete the chemokine TARC, which selectively recruits Th2 cells to the tumor [87]. Finally, Hodgkin's lymphoma sites are also enriched for immunosuppressive regulatory T cells [88]. In addition, patients with nasopharyngeal carcinoma, an epithelial cancer that is associated to 100% with EBV, carry high levels of EBV-specific IgA antibodies and have a compromised T-cell response with respect to inhibition of B-cell transformation in EBV infected PBMC cultures [89–93]. These characteristics are consistent with nonprotective Th2 polarization of the EBV-specific immune control. Finally, Burkitt's lymphoma is associated with EBV to 100% in holoendemic malaria regions. Clearance of Plasmodium falciparum from the blood is mediated by a strong Th2 polarized immune response [94, 95]. At the same time the EBV-specific immune control is compromised in Burkitt's lymphoma patients [96]. It is tempting to speculate that the Th2 cytokine milieu, which is essential to clear systemic malaria infection, does not allow a protective EBV-specific Th1 immune control to develop. Furthermore, it was found that Plasmodium falciparum infected erythrocytes prevent DC function [97] and might therefore prevent the priming of protective Th1 polarized and EBV-specific immune control.

The above listed studies suggest that EBV-specific T-cell immunity is strongly Th1 polarized, probably by DC priming, in healthy EBV carriers, but either suppressed or Th2 polarized in a nonprotective fashion in patients with EBV-associated malignancies like Hodgkin's disease, nasopharyngeal carcinoma and Burkitt's lymphoma.

43.4.2
Priming of Epstein–Barr Virus-specific Responses by Crosspresentation via Dendritic Cells Leads to Heterogeneous Affinity of T-cell Responses

Apart from the strong Th1 polarization of EBV-specific T-cell responses, priming of EBV-specific immune control by DCs could result in heterogenous affinity of EBV-specific T cells. Depending on the amount of EBV antigen taken up by DCs via B-cell fragments, DCs could prime T cells with high affinity, that would recognize EBV-transformed B cells, or with low affinity, that would hardly or not at all recognize EBV-transformed B cells.

Indeed, latent EBV antigen-specific $CD4^+$ and $CD8^+$ T-cell clones with low to undetectable recognition of EBV-transformed B cells have been described [33, 40, 98–102]. For EBNA1-specific $CD8^+$ T cells it was later shown that they recognize EBV-transformed B cells by IFNγ secretion, but only at low level by cytolysis [26, 29, 30]. However, for some EBNA1-specific $CD4^+$ T-cell clones no recognition of EBV-transformed B cells could be detected even with assays as sensitive as IFNγ ELISA [33]. These studies suggest that some low-affinity T-cell clones,-specific for latent EBV antigens, are primed via crosspresenting DCs.

In addition, it was found that EBV infected B cells undergoing lytic EBV replication display decreased MHC class I and class II surface levels [103]. Especially MHC class I was affected by a 4–5-fold downregulation. Furthermore, the late lytic EBV antigen gp42 interferes with MHC class II presentation [104] and the viral IL-10 homolog (BCRF1) downregulates important components of the MHC class I antigen processing pathway [105]. Despite these immune escape mechanisms in lytic EBV infection, both $CD4^+$ and $CD8^+$ T-cell responses against lytic EBV antigens are efficiently primed [106, 107] and even constitute the majority of expanded T cells during symptomatic acute EBV infection, called infectious mononucleosis [108, 109]. Therefore, crosspresentation of lytic EBV antigens and priming by DCs might be involved in the initiation of lytic EBV antigen-specific T-cell responses in the face of compromised MHC class I and II antigen presentation by EBV infected B cells.

Taken together, the failure of EBV-specific T cells to target EBV infected B cells might result from crosspresentation of EBV antigens by DCs in the priming of these immune responses *in vivo*.

43.5
Detection of Epstein–Barr Virus Infection by the Immune System

The above listed data suggest that DCs are involved in the priming of EBV-specific immune responses. However, it remains unknown how the immune systems detects EBV infection. Since DCs might be critical in inducing EBV-specific immune control, activation and maturation of DCs is probably a crucial checkpoint in the development of protective EBV-specific immunity. EBV infection could therefore be detected by activating receptors on DCs themselves, proinflammatory cytokine

responses by EBV infected cells or activating receptors on other innate lymphocytes, which then in turn can activate DCs.

Prominent pathogen pattern recognition receptors on DCs are Toll-like receptors (TLRs) [110]. Two of these, TLR2 and TLR9, have been reported to recognize herpesviruses, so far primarily *Herpes simplex* virus [111–113], but for TLR2 also *Cytomegalovirus* [114]. While TLR2 recognizes viral surface proteins, TLR9 seems to detect CpG motifs enriched in viral genomes [115]. The 184 kbp DNA of EBV is composed of 60 mole percent guanine and cytosine [1]. While this CG content is less than in *Herpes simplex* (71%), it might still be able to engage TLR9 for direct activation of DCs. Therefore, DCs might detect EBV infection after uptake of viral DNA in tonsils.

In addition, EBV infected B cells might activate DCs. B cells respond with a type I Interferon response to EBV infection [116, 117] and also express TLR9 [118, 119], by which they might be able to detect CpG rich motifs in EBV DNA. B-cell responses, triggered by both mechanisms, could lead to a proinflammatory environment that activates DCs during EBV infection.

Finally, other innate lymphocytes like NK cells might detect EBV infected B cells directly and secrete cytokines in response like TNFα, which will then activate DCs. EBV-transformed B cells have been described as targets for activating receptors on human NK cells [19]. Both NKG2D and the NCRs were involved in NK-cell recognition of EBV positive B-cell lymphoma lines. Therefore, EBV-transformed B cells might trigger NK cells directly in the tonsils. TNFα is one of the main cytokines, released by NK cells upon activation, and CD56^{bright} NK cells, which are enriched in tonsils, are superior producers of this cytokine [120]. TNFα is also critical for DC maturation by activated peripheral blood NK cells [52, 53]. Therefore, NK cells could sense EBV infected B cells directly and activate DCs via cytokine release.

All three detection mechanisms of viral infections might contribute to efficient immune activation after EBV infection to establish the comprehensive immune control which enables asymptomatic carriage of EBV in the majority of infected individuals.

43.6
Immunotherapeutic use of Dendritic Cells against Epstein–Barr Virus

Since DCs might initiate NK and T-cell responses to EBV during the establishment of EBV-specific immune control, they should be harnessed for immunotherapy of EBV-associated malignancies. For this purpose, DCs could be used for both generation of EBV-specific T-cell lines, which can be used for adoptive transfer therapy of passive immunotherapy, as well as for active immunization against latent EBV antigens associated with EBV positive tumors.

Adoptive T-cell therapy was successfully used against EBV-associated lymphoproliferative disease after hematopoietic progenitor cell and solid organ transplantation [11, 12, 121–127]. In addition, more recently this therapy was also used against Hodgkin's disease and nasopharyngeal carcinoma [128, 129], but with only partial success. The only partial success of adoptive T-cell therapy for Hodgkin's disease

and nasopharyngeal carcinoma might be due to the fact that autologous EBV-transformed B cells or lymphoblastoid cell lines (LCL) were used for the expansion of EBV-specific T-cell lines. These cells, however, express all latent EBV antigens and even lytic EBV antigens in a subset of EBV infected B cells, while EBV positive Hodgkin's lymphoma cells express only EBNA1, LMP1 and LMP2 and nasopharyngeal carcinoma only EBNA1 and LMP1 or EBNA1 and LMP2 [4]. T cell expansion with LCLs results in the preferential expansion of early lytic EBV antigen and EBNA3 antigen-specific $CD8^+$ T cells [130], which are unable to target Hodgkin's lymphoma nor nasopharyngeal carcinoma. In contrast, DCs can be pulsed withspecific EBV antigens or EBV antigen derived peptides for the selective expansion of EBNA1, LMP1 and LMP2-specific T cells. This approach will not only focus T-cell lines, destined for adoptive transfer, towards the-specific EBV antigens present in these tumors, but has also proven to be more efficient in the expansion of EBV-specific T cells when stimulation with DCs was compared to stimulation with EBV-transformed B cells [73, 74]. Therefore, DCs should be useful for the targeted expansion of T-cell lines against EBV-associated malignancies, that do not express all latent EBV antigens.

In addition, active immunization with peptide-pulsed DCs has been attempted for the treatment of nasopharyngeal carcinoma [131]. Peptides from the subdominant $CD8^+$ T-cell antigen LMP2 have been loaded onto autologous monocyte-derived DCs for this study. While 9/16 patients developed transient $CD8^+$ T-cell responses to the LMP2 epitopes, included in the DC vaccine, only 2/16 displayed partial regression. On the basis of similar results for DC immunotherapy of melanoma, it has been argued that stimulation of $CD4^+$ T cell help in parallel to cytotoxic $CD8^+$ T cells is required to achieve sustained antitumor T-cell responses [132]. In analogy, future EBV-specific vaccines against nasopharyngeal carcinoma will include both LMP2 for $CD8^+$ T-cell stimulation and EBNA1 for $CD4^+$ and maybe also $CD8^+$ T-cell stimulation [133]. Therefore, active immunization with DCs after loading with EBV antigens *in vitro* as well as targeting of EBV antigens to DCs *in vivo* might be promising avenues to reestablish EBV-specific immune control in patients with EBV-associated malignancies.

43.7 Summary

Although Epstein–Barr virus does not seem to infect dendritic cells directly, DCs play a critical role in the activation of NK cells, which might control B-cell transformation early after EBV infection, and in the priming of protective T-cell immune control (Fig. 43.1). This results in a strong Th1 polarization of the EBV-specific T-cell response, but might also generate some EBV-specific T cells, which due to crosspresentation of large amounts of EBV antigens by DCs have suboptimal affinity to target EBV-transformed B cells. Since DCs fulfill these crucial functions in the immune response against EBV, they should be harnessed for immunotherapy against EBV-associated malignancies. Promising trials in this direction are already underway.

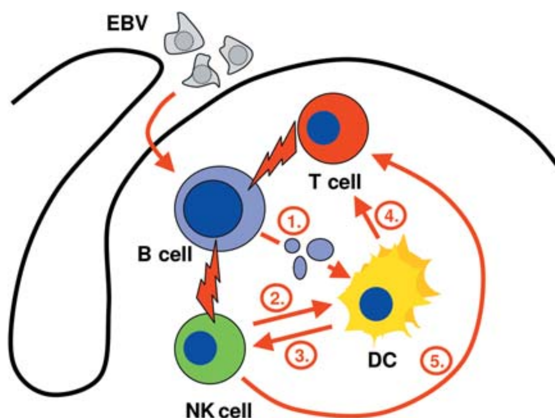

Fig. 43.1 Dendritic cells (DCs) coordinate the immune response to Epstein–Barr virus (EBV) in the tonsils. After EBV enters tonsils probably via tonsillar crypts and infects B cells, DCs sense the infection either directly through fragments of EBV-infected B cells or viral particles (1) or are matured via other innate lymphocytes like for example NK cells (2), which detect B-cell transformation by EBV. DCs then activate NK cells as a first line of defense (3) before priming EBV-specific T-cell responses (4). NK cells assist DCs in the polarization of EBV-specific T-cell responses towards protective Th1 immunity (5).

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XIV
Fungi

44
Dendritic Cells in Immunity and Vaccination against Fungi

Luigina Romani and Paolo Puccetti

44.1
Introduction

Human beings are continuously exposed to fungi, yet they rarely get fungal diseases. Although not unique among infectious agents, fungi possess complex and unusual relationships with the vertebrate immune system, partly due to some prominent features [1], among these, their ability to exist in different forms and to reversibly switch from one to the other in infection. Because cycling between different morphotypes is not obligatory for fungi as it is for other organisms, morphological transition is a mechanism fungi have evolved to adapt to different environments. This may explain why, although associations between morphogenesis and virulence have long been presumed for fungi that are human pathogens [2], no molecular data unambiguously establish a role for fungal morphogenesis as a virulence factor [3]. What fungal morphogenesis implicates, through antigenic variability, phenotypic switching, and dimorphic transition, is the existence of a multitude of recognition and effector mechanisms to oppose fungal infectivity at the different body sites [4, 5].

The need for most fungi is a stable host–parasite interaction that is achieved upon the implicit agreement that the elicited immune response be strong enough to allow host survival without pathogen elimination and to establish commensalism/persistency without excessive pro-inflammatory pathology. Therefore, the balance of pro-inflammatory and anti-inflammatory signaling is a prerequisite for successful host/fungus interaction. In light of these considerations, the responsibilities for virulence is shared by the host and the fungus at the pathogen–host interface, regardless the mode of its generation and maintenance. Studies with *Candida albicans* have provided a paradigm that incorporates contributions from both the fungus and the host to explain the theme of the origin and maintenance

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of virulence for pathogens and commensals [6]. Through a high degree of flexibility, the model accommodates the concept of virulence as an important component of fungus fitness *in vivo* within the plasticity of immune responses orchestrated by dendritic cells (DCs). Conceptually, this implies that the qualitative development of adaptive response to a fungus may not primarily depend on the nature of the fungal form being presented but rather on the type of cell signaling initiated by the ligand/receptor interaction in DCs. Therefore, the functional plasticity of DCs at the pathogen/host interface may offer new interpretative clues to fungal virulence.

44.2
Immunity to Fungi

Protective immunity against fungal pathogens is achieved by the integration of two distinct arms of the immune system, the innate and adaptive (or antigen-specific) responses [5]. The majority of fungi are detected and destroyed within hours by innate defense mechanisms [7, 8]. Most of the innate mechanisms are inducible upon infection and their activation requires specific recognition of invariant evolutionarily conserved molecular structures shared by large groups of pathogens (also known as PAMPs, pathogen-associated molecular pattern) by a set of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) [9–13]. Antigen-independent recognition of fungi by the innate immune system leads to the immediate mobilization of immune effector and regulatory mechanisms that provide the host with three crucial survival advantages: rapid initiation of the immune response (both innate and adaptive) and creation of the inflammatory and co-stimulatory context for antigen recognition; establishment of a first line of defense, which holds the pathogen in check during the maturation of the adaptive response; and steering of the adaptive response towards the cellular or humoral elements that are most appropriate for protection against the specific pathogen. Therefore, the goal to achieve the optimal activation of the antigen-specific immunity cannot be achieved without effectively activating the pathogen-detection mechanisms of the innate immune response [8].

In vertebrates, however, if the infectious organism can breach these early lines of defense an adaptive immune response will ensue, with generation of antigen-specific T helper (Th) effector and B cells that specifically target the pathogen and memory cells that prevent subsequent infection with the same microorganism. There is extensive plasticity in the T-cell response to fungi [5]. The flexible program of T cells leads to the production of many mediators, including cytokines. Due to their action on circulating leukocytes, the cytokines produced by fungus-specific T cells are instrumental in mobilizing and activating antifungal effectors, thus providing prompt and effective control of infectivity once the fungus has established itself in tissues or spread to internal organs. To limit the pathologic consequences of an excessive inflammatory cell-mediated immune reactions, the immune system resorts to a number of protective mechanisms, including the reciprocal cross-regulatory effects of Th1- and Th2-type effector cytokines, such as interferon (IFN)-

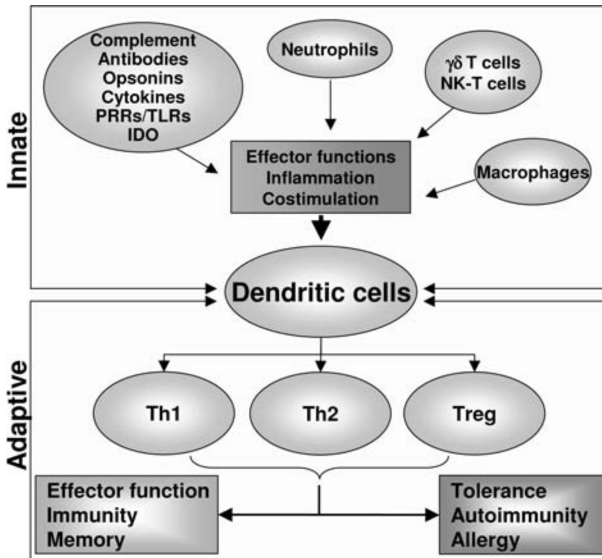

Fig. 44.1 The interface between innate and adaptive immunity to fungi. Essential to the successful removal of fungal pathogens is the early recognition of fungi by components of the innate immune system. These involve the complement system, opsonins, antibodies, IDO-dependent metabolic pathways and specialized receptors such as TLRs expressed on phagocytes and dendritic cells that recognize specific fungal derived molecular structures. Successful engagement of some of these pathways leads to an inflammatory response with destruction of the pathogen alongside the establishment of dendritic cell and T cell interactions. A well orchestrated innate and adaptive immune response will lead to pathogen radication and host immunity (left). Failure to efficiently discriminate self from nonself in innate as well as adaptive immunity can lead to pathogen proliferation and ultimately to dysregulated immunity, such as autoimmunity, allergy and sepsis (right).

γ and interleukin (IL)-4, and the generation of regulatory T cells (Treg) capable of finely tuning antifungal inflammatory and Th reactivity [5]. Therefore, host resistance to fungi seems to depend on the induction of innate and adaptive cellular immune responses that are intimately linked and controlled by sets of molecules and receptors that act to generate the most effective form of immunity for protection against fungal pathogens (Fig. 44.1). The dichotomous Th-cell model has proven to be a useful construct that shed light on the general principle that diverse effector functions are required for eradication of different fungal infections.

44.3
Dendritic Cells at the Host/Fungi Interface

As DCs are equipped with several TLRs, they are the main connectors of the innate and adaptive immune systems. In infections, they are central in the balancing act between immunopathology and protective immunity generated by host–microbe

interactions [14–16]. Studies *in vivo* suggested that DCs had the ability to internalize fungi at the sites of the infection [17, 18]. Soon after the infection, *Candida albicans* yeasts were found inside DCs from the gut and *Aspergillus fumigatus* conidia inside pulmonary DCs. In the case of *Candida*, the fungus appeared to translocate across the epithelial layers and to be subsequently phagocytosed by DCs (unpublished observations). For *Aspergillus*, DCs present in the alveolar spaces phagocytosed conidia, translocated to the space below, within the alveolar septal wall, and reached the draining lymph nodes where fungus-pulsed DCs instructed local development of antifungal Th reactivity [17]. It is known that DCs of the respiratory tract are specialized for uptake/processing but not for antigen presentation, the latter requiring cytokine maturation signals that are encountered after migration to regional lymph nodes [19]. Studies *in vitro* have shown that both human and murine DCs recognize and internalize a number of fungi, including *A. fumigatus* [17, 20–24], *C. albicans* [22, 25–31], *Cryptococcus neoformans* [32, 33], *Histoplasma capsulatum* [34], *Malassezia furfur* [35, 36] and *Saccharomyces cerevisiae* [37] and that fungi and fungal products may affect DC functioning as well [30, 31, 38, 39]. Profiling gene expression on DCs by microarray technologies has revealed that both a shared response and a pathogen-specific gene expression program were induced upon the exposure to bacteria, viruses and fungi [40]. Additional studies with *S. cerevisiae* have shown that recombinant yeast could represent an effective vaccine for the generation of broad-based cellular immune responses [37]. It seems, therefore, that DCs are uniquely able at decoding the fungus-associated information at the host/fungus interface.

44.3.1 Fungal Recognition by Dendritic Cells and Receptor Cooperativity

The DC system comprises a network of different subpopulations [16]. DC subsets differ in their phenotype, micro environmental localization, migration potential, PRR expression, responsiveness to microbes, and their capacity to induce and regulate distinct arms of the innate and adaptive immune systems [41–43]. DCs of myeloid (MDCs) and plasmacytoid (PDCs) type have been described both in mice and humans. Murine lymphoid DCs (LDCs) expressing the CD11c integrin and the CD8α antigen have also been described [16]. The ability of a given DC subset to respond with flexible activating programs to the different stimuli [43] as well as the ability of different subsets to convert into each others [44, 45] confers unexpected plasticity to the DC system. PRRs and TLRs also contribute to the functional plasticity of DCs in response to microbes. PRRs for fungi include receptors for a variety of complement components (CRs), for the Fc portion of immunoglobulins (FcRs), C-type lectins, receptors for mannosyl/fucosyl glycoconjugate ligands (MRs), for β-glucan (dectin-1), and TLRs [9–13, 46, 47].

MRs belong to a family of lectins that mediate nonopsonic phagocytosis of fungi [48]. MR ligation by fungi can be linked to induction of effector functions, but the link is dependent on activation. C-type lectins bind carbohydrates from pathogens and also self-glycoproteins, and thus they play an important role not only in

pathogen sensing but also in cell adhesion and migration. The C-type lectin DC-SIGN is widely expressed on DCs and mediates recognition of several distinct pathogens, such as viruses, bacteria and fungi [49]. The common characteristic of these pathogens is that they cause chronic infections in which the Th1/Th2/Treg balance is a critical determinant of pathogen persistency. Dectin-1 is a C-type lectin receptor that mediates attachment and ingestion of zymosan by DCs and other phagocytes [50]. Dectin-1 cooperates with TLR2 in the recognition of zymosan, by enhancing TLR2-mediated activation of nuclear factor (NF)-κB and IL-12/TNF-α production [50]. TLRs are type I transmembrane proteins that are grouped into the same gene family based on their sequence similarity. Eleven mammalian TLRs have been described so far, and TLR ligands include PAMPs and additional ligands, including endogenous ligands of host origin [51]. The ability of PAMPs to induce costimulatory molecule expression on DCs suggests a permissive role of the PAMP/TLR system in the activation of T lymphocytes during antigen presentation. All TLRs activate a core set of stereotyped responses, such as inflammation. The down-stream signaling pathway utilized by most TLRs involve the recruitment of the adapter protein MyD88 (*Drosophila* myeloid differentiation primary response gene 88) culminating in activation of NF-κB and mitogen activated protein kinases (MAPKs) that activate the transcription of the inflammatory and adaptive immune responses. However, in the case of TLR3- and TLR4-dependent signaling, other proteins may also serve as adapter molecules with or in place of MyD88 [52]. Evidence suggests that individual members of the TLR family or other PRRs interact with each other and cumulative effects of these interactions instruct the nature and outcome of the immune response to the provoking pathogen [53].

Candida and *Aspergillus* proved to be useful pathogen models to dissect events occurring at the fungus/DC interface. Murine and human MDCs and PDCs internalize *Candida* yeasts, *Aspergillus* conidia and hyphae of both (Fig. 44.2). The uptake of the different fungal elements occurred through different receptors and forms of phagocytosis. Live unopsonized yeasts, conidia or hyphae were mainly internalized through a phagocytic process. Transmission electronic microscopy indicated that internalization of yeasts and conidia occurred predominantly by coiling phagocytosis, characterized by the presence of overlapping bilateral pseudopods which led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional zipper-type phagocytosis, characterized by the presence of symmetrical pseudopods which strictly followed the contour of the hyphae before fusion. The fate of the different forms of the fungi inside cells appeared to be quite different. Two and four hours later, the majority of *Candida* [25] and *Histoplasma* [34] yeasts were found degraded inside phagosomes. In contrast, as early as one hour after infection, *Candida* hyphae appeared to escape the phagosome and were found lying free in the cytoplasm of cells [25]. For *Aspergillus*, two hours after the exposure, numerous conidia were found inside DCs with no evidence of conidia destruction, as opposed to hyphae, that were rapidly degraded once inside cells [17]. As killing of conidia would seem to be a necessary prerequisite to obtain efficient antigen presentation, it can be postulated that either a small number of conidia are actually degraded by mature DCs thus

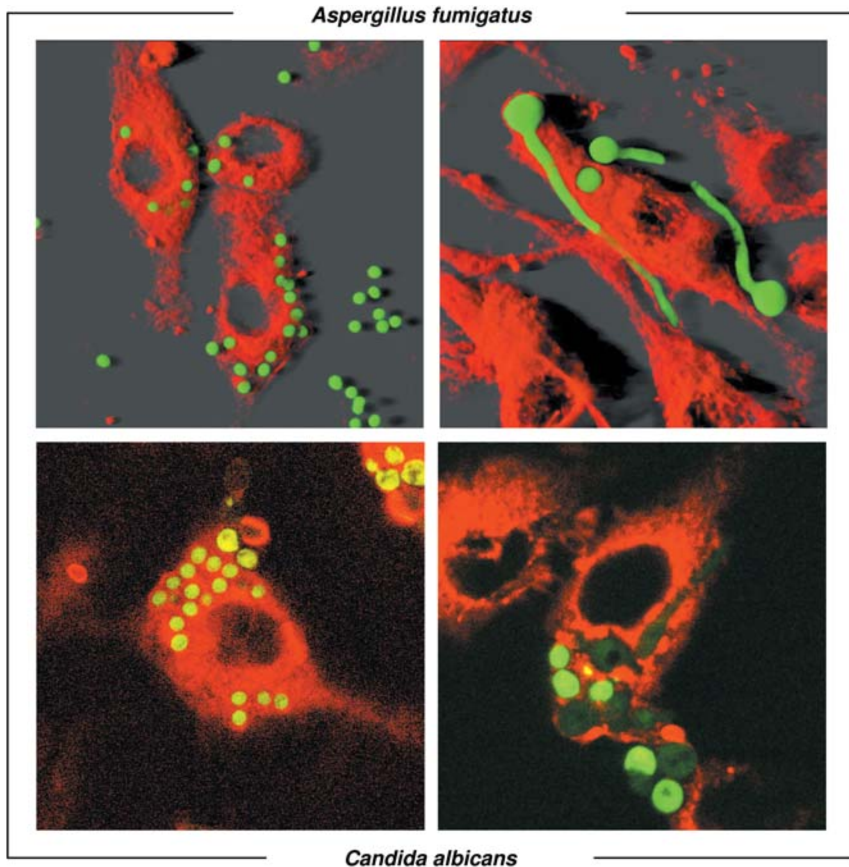

Fig. 44.2 Murine dendritic cells phagocytose different fungal morphotypes. The pictures illustrate a three-dimensional reconstruction of confocal microscopy sections of fetal skin-derived dendritic cells phagocytosing *Aspergillus fumigatus* conidia and hyphae (top panel) or *Candida albicans* yeasts and hyphae (lower panel). Reconstruction was performed with the isosurface module of Imaris software (Bitplane) on a SGI octane workstation. Cells were stained with the DiQ vital stain. GFP-expressing *Aspergillus* was kindly provided by Margo M Moore (British Columbia Canada) and GFP-expressing *Candida* by Joachim Morschhäuser (Würzburg Germany).

allowing their antigen processing and presentation or, alternatively, antigens could be processed and regurgitated by other infected phagocytes and then transferred to DCs for presentation.

Recognition and internalization of unopsonized yeasts and conidia occurred through the engagement of MRs of different sugar specificity, DC-SIGN, dectin-1 and, partly, CR3 [24–26, 28, 29]. In contrast, entry of hyphae occurred by a more conventional, zipper-type phagocytosis and involved the cooperative action of FcγR II and III and CR3 [29]. Phagocytosis does not require TLR2, TLR4, TLR9 and MyD88 [54]. For other yeasts, such as those of *Cryptococcus*, a cooperative interac-

tion between MR and FcγR has been shown [32], whereas *Histoplasma* yeasts were found to be phagocytosed through the fibronectin receptor, very late antigen-5 [34]. Consistent with the findings that signals from protein kinase C (PKC) and/or protein tyrosine kinases are required for phagocytosis in a variety of systems [55], the PKC inhibitor staurosporine was required for CR- and FcγR-mediated phagocytosis, while FcγR- and, to a lesser extent, MR-mediated phagocytosis required signaling through protein tyrosine kinases [56]. The results are consistent with the view that fungi have exploited common pathways for entry into DCs, which may include a lectin-like pathway for unicellular forms and opsono-dependent pathways for filamentous forms. In terms of sugar specificity, this may vary among fungi, as DCs recognize *Candida* yeasts through a mannose-fucose receptor [26] and *Aspergillus* conidia through a lectin receptor of galactomannan specificity [24, 57]. Actually, the sugar specificity of MRs involved in the entry of one or multiple *Aspergillus* conidia turned out to be different, as the entry of multiple conidia occurred through a pathway sensitive to galactomannan and that of one single cell through a pathway sensitive sensitive to β-glucan [17]. Therefore, fungal surface polysaccharides have a key role in the DC/fungi interactions. It also appears that unicellular fungal forms may exploit the CR3 receptor on DCs as a niche to avoid degradation through the multilectin pathway while allowing their own persistence [58].

44.3.2 Dendritic Cell Activation

The engagement of distinct receptors by different fungal morphotypes translated into downstream signaling events, ultimately regulating cytokine production, costimulation and fungus survival. Entry of *Candida* yeasts or *Aspergillus* conidia through MRs and dectin-1 resulted in the production of pro-inflammatory cytokines, including IL-12, upregulation of costimulatory molecules and histocompatibility Class II antigens. IL-12 production by DCs required the MyD88 pathway with the implication of distinct TLRs (IL-1RI and TLR9 for *Candida* and TLR4 and TLR9 for *Aspergillus*) [54, 59]. These events were all suppressed upon entry through CR3. In contrast, coligation of CR3 with FcγR, as in the phagocytosis of hyphae, resulted in the production of IL-4/IL-10 and upregulation of costimulatory molecules and histocompatibility Class II antigens [6, 56]. The production of IL-10 was largely MyD88-independent [23, 54]. Therefore, TLRs collaborate with other innate immune receptors in the activation of DCs against fungi through MyD88-dependent and -independent pathways (Fig. 44.3). It is of interest that TLR gene expression on DCs could be affected upon fungal exposure in a morphotype-dependent manner [22] and that the TLR9 agonist CpG-ODN could convert an *Aspergillus* allergen to a potential protective antigen [60]. These observations points to the potential for TLR agonists to act upon the degree of flexibility of the immune recognition pathways to fungal antigens and allergens.

It is known that MAPKs participate in signal transduction events associated with a number of stimuli, such as mitogens, growth factors, and pathogen-derived products [61]. p38 and JNK 1/2 MAPKs are known to regulate IL-12/IL-10 expression on

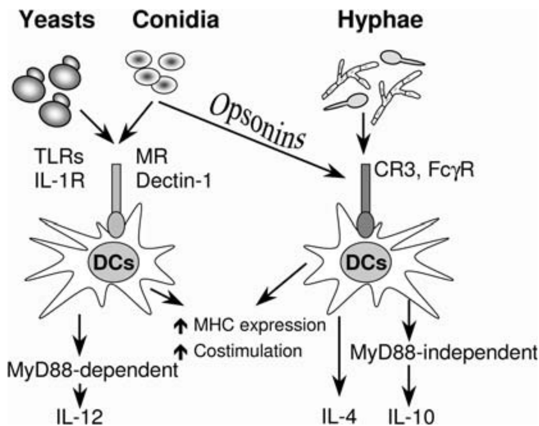

Fig. 44.3 The exploitation of distinct recognition receptors in dendritic cells by *Candida albicans and Aspergillus fumigatus.* Distinct, multiple recognition receptors on dendritic cells (DCs) are activated by fungi. The engagement of distinct receptors translated into downstream signaling events that differentially affect cytokine production. Fungal opsonins may subvert the receptor exploitation by fungal morphotypes. The exploitation of a specific receptor invariably leads to the occurrence of a specific type of Th cell reactivity.

DCs [62]. Our own data suggest that the production of IL-12/IL-10 in response to yeasts and hyphae of *C. albicans* is directly associated with the activation/inhibition of certain MAPKs. The production of IL-12 in response to yeasts was associated with the selective inhibition of extracellular signal-regulated kinases (ERK 44/42) while the downregulated production of IL-12 and upregulated production of IL-10 in response to hyphae was associated with the activation of ERK 44/42 and the selective inhibition of p38MAPKs (Bonifazi P., personal communication). These data are consistent with the notion that ERK suppresses the induction of IL-12 and enhances IL-10 production in DCs [62] and parallel similar data obtained with human monocytes exposed to *C. albicans* [63].

In terms of survival, fungi were rapidly degraded upon entry through MR or FcγR, a finding in line with the notion that ligation of these receptors is usually sufficient to trigger a vigorous oxidative burst and generation of pro-inflammatory signals on innate phagocytes [8]. For CR3, it is of interest that *Candida* exploited this receptor to survive inside DCs [6], while *H. capsulatum* used this receptor to survive in macrophages [64] but not in DCs [34]. This is consistent with the observation that CR3 engagement is one most efficient uptake of opsonized fungi but it has the remarkable characteristic of a broad capacity for recognition diverse fungal ligands. In this regard, it is worth mentioning that *C. albicans* possesses fungal molecules mediating interaction with CR3 but avoiding production of nitric oxide [8]. The multiplicity of binding sites and the existence of different activation states enables CR3 of disparate (both positive and negative) effector activities against fungi [9].

A remarkable and important feature of Peyer's patches (PP)-DCs is the production of IL-10 in response to *Candida*, an event occurring by signaling through CR3 in the presence of opsonizing antibodies. These IL-10-producing PP-DCs activate $CD4^{+}CD25^{+}$ Treg that negatively affect antifungal Th1 reactivity [65, 66]. It is conceivable that tissue-dependent factors and opsonins (see below) may modulate receptor usage by DCs at different body sites, thus contributing to the functional plasticity of DCs at the host/pathogen interface.

It has recently been shown that fungal RNA acts as potent DC activator [67]. Although extracellular mRNA induced DC activation by signaling through a nucleotide receptor [68], fungal RNA also activated TLR expression on DCs [22]. Upon exposure to fungal RNA, DCs underwent functional maturation, as indicated by the upregulated expression of costimulatory molecules and MHC class II antigens and cytokine production [21, 27].

44.3.3 Dendritic Cell Conditioning

44.3.3.1 Opsonins

Fungal opsonins are known to affect the uptake of fungi by phagocytic cells [8]. Opsonization with mannose binding lectin (MBL), C3 and/or antibodies subverted the receptor exploitation on DCs by the different fungal morphotypes and, ultimately, affected DC activation [29]. MBL, a member of the collectin family of proteins, bind through multiple sites to various carbohydrate structures on fungal surfaces and promote complement activation through the lectin pathway [69]. Opsonization with MBL or C3 and/or or IgG greatly modified the receptor exploitation by fungi [29]. Opsonization with MBL and C3, by favoring entry through CR3, reduced the expression of costimulatory molecules and IL-12 production, while C3 and IgG opsonization, by favoring the entry through CR3 and FcγR, significantly reduced production of IL-12, increased that of IL-4 and induced that of IL-10. Thus, collectins appear to favor the phagocytosis of the fungus without implicating the production of cytokine messengers to the immune system, an activity compatible with a primitive mechanism of host defense and in line with their ability to downregulate the inflammatory response to fungi [69]. Antifungal antibodies also modified the receptor usage and DCs activation in response to fungi [18]. Opsonization with protective anticandidal antibodies, specifically reacting to the phosphomannan protein complex of *C. albicans* [18], while not affecting the phagocytosis, greatly affected the fungal internalization through CR3 and increased IL-10 production by PP-DCs, a finding consistent with the notion that the protective potential of opsonizing IgM antibodies may rely on their ability to fix complement C3 on the fungal surface. All together, opsonins, by subverting the morphotype-specific program of activation of DCs, may qualitatively affect DC functioning in response to fungi.

44.3.3.2 Tryptophan Metabolic Pathway

Recent evidence suggest that the inflammatory/anti-inflammatory state of DCs in response to fungi is strictly controlled by the metabolic pathway involved in tryptophan catabolism and mediated by the enzyme indoleamine 2,3-dioxygenase (IDO) [70]. IDO has a complex role in immunoregulation in infection, pregnancy, autoimmunity, transplantation, and neoplasia [71]. IDO expressing DCs are regarded as regulatory DCs specialized to cause antigen-specific deletional tolerance or otherwise negatively regulating responding T cells [72]. IFN-γ is required for functional IDO enzymatic activity in DCs [73, 74].

In candidiasis, IDO activity was induced at sites of infection as well as in DCs via IFN-γ- and cytotoxic T lymphocyte-associate antigen (CTLA) 4-dependent mechanisms. IDO inhibition greatly exacerbated the infection and associated inflammatory pathology, as a result of deregulated innate and adaptive immune responses. *In vitro*, IDO blockade reduced IL-10 production in response to hyphae and increased IL-6/IL-12 production in response to yeasts by PP-DCs. Consistent with the finding that PP-DCs producing IL-10 are absolutely required for the activation of $CD4^+$ $CD25^+$ Treg capable of negatively regulating the inflammatory response and antifungal Th1 immunity upon adoptive transfer *in vivo* [66], the number of IL-10-producing $CD4^+$ $CD25^+$ Treg was significantly decreased in mice with candidiasis upon IDO blockade. Concomitantly, the number of $CD4^+$ T cells producing IFN-γ increased, while that of cells producing IL-4 would decrease. It appears that the activation of IL-10-producing $CD4^+$ $CD25^+$ T cells is one important mechanism through which the IFN-γ/IDO-dependent pathway may control the local inflammatory pathology and Th1 reactivity to the fungus. These results provide novel mechanistic insights into complex events that, occurring at the fungus/pathogen interface, relate to the dynamics of host adaptation to the fungus. The production of IFN-γ may be squarely placed at this interface, where IDO activation likely exerts a fine control over inflammatory and adaptive antifungal responses (Fig. 44.4). Therefore, the selective expression of IDO in the gut may represent the missing tissue-dependent factor that conditions the ability of DCs to produce IL-10 upon exposure to *Candida* hyphae, ultimately dictating the local pattern of both cytokine production and Th reactivity to the fungus. In addition, as *C. albicans* is a commensal of the human gastrointestinal and genitourinary tracts and IFN-γ is an impor-

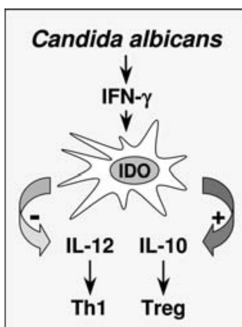

Fig. 44.4 The crucial role for the IFN-γ/IDO-dependent metabolic pathway in *Candida albicans* infection. The production of IFN-γ is squarely placed at the host/pathogen interface where IDO activation exerts a fine control over dendritic cell activation and the resulting adaptive immunity to the fungus.

tant mediator of protective immunity to the fungus [5], the IFN-γ/IDO axis may accommodate fungal persistence in a host environment rich in IFN-γ. In its ability to downregulate antifungal Th1 response in the gastrointstinal tract, IDO behaves in a fashion similar to that described in mice with colitis where IDO expression correlates with the occurrence of local tolerogenic responses [75]. In the basal state, the gut is the site of the highest levels of IDO expression which is believed to be required to allow for microbial colonization and ingestion of dietary antigens without tissue-damaging inflammatory responses. The expression of IDO is indeed upregulated in active inflammatory bowel disease [76].

44.3.3.3 **T-cell Ligands**

Bidirectional signaling through the B7-CTLA-4 co-receptor pair has been shown to down-regulate immune responses and favor tolerance to alloantigens as well as tumor and self peptides, providing a mechanistic clue to the action of CTLA-4-expressing Treg [77]. Recent data showed that both CD28 forward signaling and B7 reverse signaling positively affect the induction of immune responses after B7 engagement by CD28, providing an unexpected symmetry to the actions of CTLA-4 and CD28 in respective negative and positive costimulation of T cells. In particular, using soluble CD28-Ig and CTLA-4-Ig evidence has been provided that the two fusion proteins exert opposite effects on DC activating programs in response to *C. albicans*. This translated in a different vaccinating ability of conditioned DCs when used as a vaccine preparation. Protective vaccination with yeast-pulsed DCs was rendered nonprotective by CTLA-4-Ig, which required IFN-γ and resulted in a Th2-dominated overwhelming infection. In contrast, nonprotective DCs pulsed with hyphae were made protective by exposure to CD28-Ig, through mechanisms contingent on autocrine IL-6. Compared with that of mice receiving DCs pulsed with hyphae in the absence of CD28-Ig, the frequency of IFN-γ-producing T cells was increased and that of IL-4-producing cells was decreased, as the mice developed durable anticandidal protection [77]. Therefore, different ligands of B7, through different cytokine responses in target DCs, may induce qualitatively different Th cell responses to fungi.

44.3.3.4 **Other Cells**

A reciprocal activating interaction between Natural Killer (NK) cells and DCs has been suggested to play a role in the functional regulation of these cells in immunity to infections [78]. A study in patients with atopic eczema/dermatitis syndrome (AEDS) has shown a close contact between NK cells ($CD56^+/CD3^-$) and $CD1a^+$DCs *in vivo*, in biopsies from *Malassezia* atopy patch test-positive skin [79]. DCs prestimulated with the yeast were less susceptible to NK cell-induced cell death and soluble yeast-derived factors decreased the cytotoxic potential of NK cells. Therefore, these findings indicate that an interaction may occur between NK and DCs in the skin of AEDS patients, upon which the fungus may exert a fine control.

44.4 Dendritic Cells Translate Fungus-associated Information to Th1, Th2 and Treg Cells

Fungus-pulsed DCs activated different types of naive $CD4^+$ Th cells *in vitro* and *in vivo* [6, 22, 80, 81]. *In vitro*, $CD4^+$ T murine splenocytes co-cultured with yeast- or conidia-pulsed DCs produced high levels of IFN-γ, but not IL-4 or IL-10. In contrast, DCs exposed to hyphae induced low levels of IFN-γ, but high levels of IL-4 and IL-10 in $CD4^+$ T cells. Monocyte-derived human DCs also activated different types of cytokine-producing cells upon pulsing with the different fungal morphotypes [21, 29, 82]. Interestingly, upon pulsing with yeasts or conidia, MDCs mainly activated IFN-γ-producing $CD4^+$ Th1 cells, whereas PDCs activated IFN-γ- and IL-10-producing $CD4^+$ cells [82]. *In vivo*, the balance among the different DC subsets determined whether protective or nonprotective antifungal cell-mediated immune responses developed [80]. Langerhans cells, MDCs, and LDCs were present in the draining lymph node of mice immunized with protective or nonprotective cryptococcal antigen [80]. Draining lymph node LDC:MDC ratios induced by the protective immunogen were significantly lower than the ratios induced by either immunization in which the nonprotective immunogen was present. In contrast, mice given the nonprotective immunogen had LDC:MDC ratios similar to those of naive mice. Therefore, Langerhans cells and MDCs were needed for induction of the protective response, whereas LDC acted as negative regulators of cell-mediated immune responses.

Fungus-pulsed DCs activated different $CD4^+$ Th cells upon adoptive transfer into immunocompetent mice [21, 22, 27]. Adoptive transfer of purified *ex-vivo* DCs pulsed with yeasts/conidia or hyphae, resulted in priming of $CD4^+$ T cells for Th1 or Th2 cytokine production, respectively. The analysis of antigen specific proliferation and cytokine production by $CD4^+$ T cells from draining lymph nodes and spleens revealed that levels of IFN-γ were higher, and those of IL-4 lower, in mice immunized with yeast- or conidia-pulsed DCs as compared to mice receiving unpulsed or hypha-pulsed DCs. The ability of fungus-pulsed DCs to prime for Th1 and Th2 cell activation upon adoptive transfer *in vivo* correlated with the occurrence of resistance and susceptibility to the infections [21, 22, 27]. Antifungal protective immunity *in vivo* was also observed upon adoptive transfer of *ex-vivo* DCs transfected with fungal RNA. The efficacy was restricted to DCs transfected with RNA from yeasts or conidia but not with hyphal RNA. The effect was fungus-specific, as no cross-protection was observed upon adoptive transfer of DCs pulsed with either fungal species. It is of interest that DCs transfected with RNA from *C. neoformans*, an opportunistic fungus on occasion, also induced protection in a murine model of pulmonary cryptococcosis [22]. The frequency of IFN-γ-producing Th1 cells was increased and that of IL-4-producing cells decreased in protected mice, a finding suggesting the occurrence of a Th1-dependent antifungal resistance. It is of interest that yeast or conidial RNA, more efficiently than live fungi, concurrently activated IL-10-producing Treg (Table 44.1). These findings expand upon the vaccinating potential of DCs in fungal infections.

Tab. 44.1 DC vaccination against fungi.

In vitro		*In vivo*			
DC pulsed with:	***Th priming***	***Frequency of T cells producing:***			***Ref.***
		IFN-γ	***IL-4***	***IL-10***	
Candida yeasts	Th1	↑	↓		25
Aspergillus conidia	Th1	↑	↓		21
Yeasts/conidial RNA	Th1+Treg	↑	↓	↑	21, 27
Cryptococcal RNA	Th1+Treg	↑	↓	↑	22
Aspergillus hyphae	Th2	↓	↑	↑	20
Candida hyphae	Th2	↓	↑	↑	21
Hyphal RNA	Th2	↓	↑	↑	21, 27

↑ or ↓, increased or decreased compared to controls.

44.5 Exploiting Dendritic Cells as Fungal Vaccines

The infusion of RNA-transfected DCs accelerated the recovery of functional antifungal Th1 responses in mice with allogeneic hematopoietic stem cell transplantation (HSCT), an experimental model in which autologous reconstitution of host stem cells is greatly reduced to the benefit of a long-term, donor type chimerism in more than 95% of the mice and low incidence of graft versus host disease [83]. Patients receiving T cell-depleted HSCT are unable to develop antigen-specific T cell responses soon after transplant [84] and showed a defective DC functioning [85]. However, functional recovery of the T cell system after T cell-depleted allogeneic HSCT has been demonstrated [86] and both donor and recipient DCs may participate to the reconstitution of the T cell repertoire in transplantation through distinct pathways of antigen presentation [87]. We have demonstrated that an imbalanced production of Th1 and Th2 cytokines was responsible for the susceptibility to fungal infections in the murine HSCT model [83]. However, readdressing the balance between Th1 and Th2 subsets, as by treatment with Th2 cytokine antagonists, accelerated the recovery of Th1-mediated antifungal resistance [83]. The recovery of functional Th1 cells producing IFN-γ was also accelerated by the infusion of fungus-pulsed or RNA-transfected DCs, a finding suggesting that DCs may pivotally determine the Th/Treg balance in HSCT [21, 27]. We have also found that the ability of either MDCs or PDCs to phagocytose and respond to *Candida* or *Aspergillus* was defective soon after allogeneic HSCT (unpublished data). In contrast, both murine and human donor MDCs and PDCs phagocytosed fungi and underwent functional maturation in response to them. However, their activation program for cytokine production was different, being IL-12 produced mainly by MDCs and IL-12, IL-10 and IFN-α produced by PDCs. This resulted in a distinct ability for T cell

priming *in vitro*, being Th1, Th2 and Treg differently activated by the different DC subsets [82]. More recent data have shown that the infusion of fungus-pulsed purified DCs of either subset accelerated the recovery of peripheral antifungal Th1 immunity and increased resistance to fungal infections in mice with HSCT. However, only the co-infusion of DCs of both subsets resulted in: i) induction of Treg capable of a fine control over the inflammatory pathology; ii) tolerization toward alloantigens and iii) diversion from alloantigen-specific to antigen-specific T cell responses in the presence of donor T lymphocytes (unpublished observations). Thus, the adoptive transfer of DCs may restore antifungal immunocompetence in HSCT by contributing to the educational program of T cells through the combined action of activating and tolerizing DCs. These results, along with the finding that fungus-pulsed DCs could reverse T cells anergy of patients with fungal diseases [20, 88], may suggest the utility of DCs for fungal vaccines and vaccination.

44.6 Conclusions and Perspectives

In the past decades, the frequency of opportunistic fungal infections has increased [89]. The increasing number of susceptible hosts, the introduction of newer modalities for HSCT, the evolution of organ transplantation practices, the use of novel immunosuppressive agents, and current antimicrobial prophylactic strategies have likely contributed to the changing epidemiology of invasive mycoses. The therapeutic efficacy of antifungals is limited without the help of host immune reactivity. Various cytokines, including chemokines and growth factors, have proved to be beneficial in experimental and human refractory fungal infections [90, 91]. The Th1-Th2 balance itself can be the target of immunotherapy [92, 93]. The inhibition of Th2 cytokines, or the addition of Th1 cytokines, can increase the efficacy of antifungals, such as polyens and azoles, in experimental mycoses [94].

In the past decade, a dramatic shift has occurred in our mechanistic understanding of innate immunity. Precisely, the appreciation that activation of the innate immune system initiates, amplifies and drives antigen-specific immune responses together with the identification of discrete cell types, specific receptors and the signaling pathways involved in the activation of innate immunity has provided a multitude of new targets for exploitation by the developments of adjuvants for vaccines [95]. Developments in DC biology are providing opportunities for improved strategies for the prevention and management of fungal diseases in immunocompromised patients. The model has brought dendritic cells to center stage as promising targets for intervention for immunotherapy and vaccine development and has shifted the emphasis from the “antigen” towards the “adjuvant”. The ultimate challenge will be to design fungal vaccines capable of inducing optimal immune responses by targeting specific receptors on DCs. This will require, however, further studies aimed at elucidating the convergence and divergence of pathways of immune protection elicited in infections or upon vaccination.

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Abbreviations

AEDS	atopic eczema/dermatitis syndrome
CR	complement receptor
CR3	complement receptor 3
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
FcγR	receptor for the Fc portion of immunoglobulin
FcR	Fc receptor
HSCT	hematopoietic stem cell transplantation
IDO	indoleamine 2,3-dioxygenase
IL	interleukin
IL-1R	IL-1 receptor
LDC	lymphoid dendritic cell
MAPK	mitogen-activated protein kinase
MDC	myeloid dendritic cell
MHC	major histocompatibility complex
MR	mannose receptor
MyD88	*Drosophila* myeloid differentiation primary response gene 88
NK	natural killer cell
PAMP	pathogen-associated molecular pattern
PDC	plasmacytoid dendritic cell
PKC	protein kinase C
PRR	pattern recognition receptor
Th	helper T cell
TLR	Toll-like receptor
Treg	regulatory T cell

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XV
Autoimmunity

45
Dendritic Cells in Autoimmune Diseases

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45.1
Introduction

The immune system evolved to protect us from microorganisms. The antigen (Ag)-nonspecific innate immunity and Ag-specific adaptive immunity act in concert to eradicate pathogens, using cells such as macrophages, granulocytes, dendritic cells (DCs) and lymphocytes, and different molecules such as cytokines, antimicrobial peptides, complement and antibodies [1–3]. The intrinsic complexity of the immune system renders it prone, in certain circumstances, to turn against the organism itself, thus causing the autodestruction of target organs. A few individuals are clearly genetically predisposed for such a dysfunction. Autoimmune diseases affect about 5% of individuals in developed countries. Their incidence has greatly increased over the past few years [4–7].

T- and B-cell activation and differentiation during the immune responses against foreign Ag are initiated and controlled by DCs [8–11]. Accumulating evidence also suggests that DCs play a critical role in establishing tolerance [12, 13]. Therefore DCs are the critical decision-making cells orchestrating the immune response. We propose that an intrinsic or extrinsic dysfunction of DCs lead to autoimmunity, i.e. an immune response directed against self-Ag and its resultant detrimental effects, i.e. tissue inflammation and destruction [14–21]. This chapter will review the actual knowledge of the involvement of DCs in human autoimmune diseases.

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45.2 Dendritic Cells

Prior studies have emphasized the capacity of DCs to detect infection and induce pathogen specific immune response [22–26]. In human biology, two major DC pathways are thought to exist: (1) a myeloid pathway (generating at least two subsets, including Langerhans cells (LCs), found in stratified epithelia, and interstitial DCs (intDCs), found in all other tissues) and (2) plasmacytoid DCs (pDCs) [10, 11].

After a tissue invasion by pathogens, immature and antigen-capturing myeloid DCs (mDCs) resting in peripheral tissues sense pathogens, tissue necrosis, and local inflammation via a panel of receptors which specifically recognize microbial products [27–30], pro-inflammatory cytokines such as interferon (IFN), tumor necrosis factor-a (TNF-a), interleukin 1 (IL-1) or CD40 [10, 31]. These signals induce DCs to undergo a maturation process while remodeling their expressed chemokine receptors, secreting a great amount of cytokines (i.e. IL-12) and chemokines and migrating along the afferent lymphatics into the T-cell areas of the draining lymph nodes. There, they highly express membrane associated T-cell stimulatory and binding molecules such as members of the B7 family (i.e. CD86) or TNF family receptors (i.e. CD40), they present processed Ags to T cells via both classical and nonclassical antigen-presenting molecules. This results in T-cell proliferation and differentiation into helper and effector cells [9–11]. The unique property of DCs, when compared to other antigen-presenting cells, is their optimal ability to prime naïve T cells [32]. DCs also activate B cells [33, 34], NK cells [35], and NK T cells [36], and T regulatory cells [37, 38]. T Regulatory cells constitute a small fraction of T cells which suppress the response of the other T cells even after powerful stimuli [39–43]. They arise during T-cell ontogenesis in the thymus (e.g. $CD4^{+}CD25^{+}$ Treg cells) and later on from the adaptive immune response (e.g. Tr1 cells) [43–45].

Meanwhile, plasmacytoid DCs (pDCs) resting in lymph nodes or circulating in blood secrete large amounts of IFN-$\alpha\beta$, highly potent adjuvant molecules for the entire immune response, upon exposure to viruses as well as bacterial DNA [46–49]. Importantly, these cells subsequently differentiate into DCs able to induce also an immune response [34, 50–53].

45.3 Dendritic Cells and Tolerance

Maintenance of tolerance to self antigens is presumed to reflect a combination of central and peripheral tolerance. DCs are now thought to play a pivotal role in the control of tolerance [12, 54–56].

45.3.1 Central Tolerance

The process of clonal selection is a central feature of the immune system. High-affinity self reactive T cells are usually deleted in the thymus [57–60] (Fig. 45.1). DCs

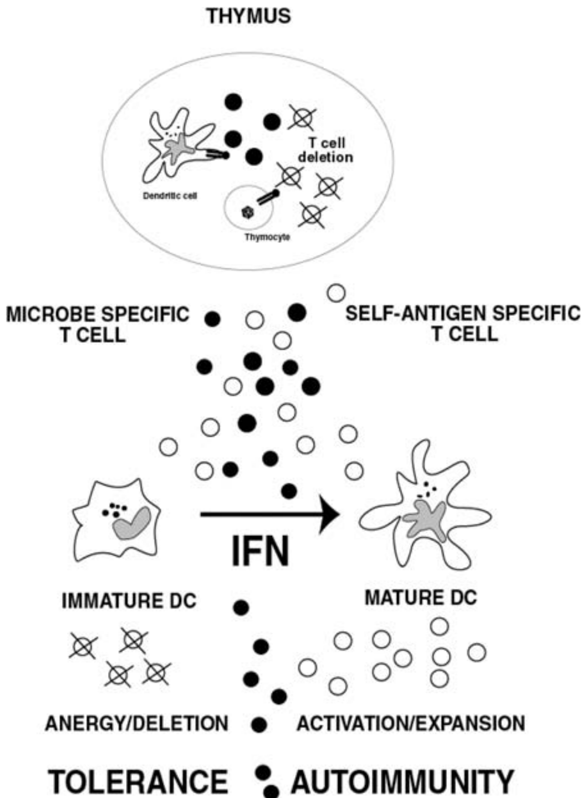

Fig. 45.1 Dendritic cells determine the fate of foreign antigen specific and self antigen specific T cells. Thymic escapees specific to self antigens are kept silent in periphery via immature DCs, which control peripheral tolerance. Excess IFN-$\alpha\beta$ induces unabated DC maturation, which leads to activation/expansion of self-antigen specific T cells.

resting in the medulla may be involved in this process. Indeed, DCs are able and sufficient to induce negative selection of autoreactive CD4+ thymocytes by clonal deletion [61–63]. Meanwhile, a few lymphocytes, interacting with high-affinity with self-antigens, differentiate into natural regulatory cells ($CD4^+CD25^+$ Treg) instead of being eliminated.

The process of B cells clonal selection takes mainly place in the bone marrow, where autoreactive B-cell disappear either by deletion or receptor editing [64–68].

However autoreactive T and B cells can escape from negative selection. Furthermore certain self or environmental Ags are not expressed in the thymus or the bone marrow. Thus many autoreactive T and B cells are going to arise from primary lymphoid organs [69–72].

45.3.2
Dendritic Cells and the Control of Peripheral Tolerance

If all individuals appear to harbor some potentially autoreactive lymphocytes not deleted in the thymus or in the bone marrow, these cells remain innocuous unless activated [73–77]. This requires that self-reactive T cells have to be kept out of an activation process in the secondary lymphoid organs. Means exploited to induce or maintain a peripheral tolerance are: ignorance, anergy, clonal deletion, phenotype/function skewing and suppression [78, 79].

There is a more difficult challenge to overcome for the immune system: DCs in steady state or during an infection constantly uptake and present self-antigens to T cells (e.g. apoptotic cells constantly produced in nonlymphoid and lymphoid organs) [80–84]. The possibility that presentation of self Ag by DCs is sufficient to induce autoimmunity was several times reported in mouse models such as systemic lupus erythemathosus (SLE), insulin-dependent diabetes mellitus (IDDM), experimental autoimmune encephalomyelitis (EAE), autoimmune myocarditis and thyroiditis. In these models, the capacity of self-Ag-loaded DCs to initiate a chain of events leading to specific autoimmune diseases has been clearly demonstrated [85–88]. The way the immune system deals with such a great danger and how it can fail and bring up autoimmunity is now starting to be understood.

There is now evidence that *immature/steady* state DCs play an important role in silencing self-reactive T lymphocytes, in other word in controlling the peripheral tolerance (reviewed in [12]). Immature/steady state DCs, in the absence of any kind of inflammation, express low levels of MHC class I and II, co-stimulatory molecules and cytokines. They are not enough differentiated to carry out their roles as inducers of the immunity. Yet, within peripheral tissues, they capture self-Ags, dying and apoptotic cells and innocuous exogenous proteins, i.e. proteins defining the immunological self. They migrate through lymphatics to the draining lymph node. There, they present self-peptide-MHC complexes, in the absence of co-stimulation signals, to silence circulating naïve autoreactive T cells either by deleting them [89–92] or by inducing the expansion and differentiation of T cells that regulate or suppress other T cells (e.g. in some circumstances the IL-10-producing Tr1 cells) [38, 42, 93–97]. For example, *in vivo*, DCs alone are efficient to crosspresent self Ag to CD8 T cells [98] and may be responsible for the induction of tolerance seen after a wave of apoptosis in the pancreas [99]. Selective depletion of DCs has also been showed to block the depletion of Ag-specific T cells by Ag loaded dying splenocytes [92]. By suppressing a mandatory T-cell help, DCs may also avoid a self-Ag specific and T-dependent B-cell activation. In contrast, a concomitant activation with anti-CD40 results in the mounting of a potent immune response, as DCs are induced to express a large number of co-stimulatory molecules [90]. This theory was somehow challenged by the need for the following prerequisites: immature/steady state DCs have to (i) display capacity to migrate into the lymph nodes and (ii) efficiently process and present self Ag on both MHC class I and II. Both prerequisites, which had not been demonstrated *in vitro* or *ex vivo,* have been now clearly demonstrated *in vivo*. Indeed, immature DCs are in steady state the main

population of DCs in the lymph nodes [100, 101], where they constitute the long recognized afferent flux of veiled cells [102]. Apoptotic cells do not induce DCs maturation like necrotic cells, but still induce the expression of the lymph node homing receptor CCR7 [103]. Inside the draining lymph nodes of the intestinal epithelium, DCs are loaded with self Ag, e.g. apoptotic cells or the gastric H^+/K^+-ATPase [83, 104]. In the same manner, DCs capture airway, oral, pancreatic and skin Ags even without any ongoing infection [99, 105–107]. In the mouse, the CD8+ DC subset, which equivalent is not clearly identified in humans, seems to be specifically dedicated for uptaking apoptotic cells [108].

Finally, immature/steady state DCs *in vivo* efficiently process and present Ag to T cells inducing tolerance [89–91, 109]. In conclusion, during the steady state, immature DCs purge the T-cell repertoire from its dangerous self-specific components, deleting them and eliciting T-cell suppression, protecting us from autoimmunity which otherwise would arise later on, when activated DCs will present self-Ag in an immunogenic manner. It is easy to figure out how this system may have its failures. For example, an "inappropriate" activation of a previous immature/steady state/tolerogenic DC, before that every peripheral autoreactive T-cell have been tolerized, may launch immunity against self-Ag.

If *mature* DCs are geared toward the launching of Ag-specific immunity, they also can induce the expansion and differentiation of T cells which regulate or suppress other T cells [12, 18]. This probably involves the CD4+CD25+ Treg and may be crucial to limit the collateral damages occurring during an infection or a nonspecific inflammation, when DCs are obviously activated.

The molecular mechanisms underlying the tolerogenic properties of peripheral DCs are now starting to be elucidated and may involve several intricated mechanisms: (a) lack of co-stimulation; (b) cell death induction by expression of indoleamine 2,3-dioxygenase (IDO) which induces the catabolism of tryptophan, or by Fas/Fas-L interaction; (c) secretion of IL-10/TGF-b; (d) signaling through CD80/CD86 and CTLA-4 or PDL1/2 and PD-1 or Immunoglobulin-like transcript 3 (ILT3) and ILT4 [18, 78, 110].

45.4 Dendritic Cell Activation and the Priming of Autoimmune Diseases

We will discuss first a threshold model where the autoimmune response comes from an excessive presentation of self peptides by DCs, leading to autoreactive T-cell activation. This excess comes either from a bystander activation of DCs, or a defective downregulation of activated DCs. In this model, the number of self-Ag loaded an efficient DCs and the duration of self-Ag presentation by DCs exceed the regulatory mechanisms to elicit an activation of self reactive lymphocytes. We will then consider how, during an immune reaction, the emergence or the lack of certain DC subsets may skew the activation/differentiation of T cells clones, eventually leading to autoimmunity: the rise of "autoimmune-prone" DC subsets. These theories include failures, relative or absolute, of the regulatory mechanisms de-

signed to counteract autoreactive T and B cells activation, which we will not discuss here [111–113].

45.4.1 Autoimmunity through Bystander Activation of Dendritic Cells

It is now established that normal DCs can increase the presentation of self-Ag (see above). Thus, it is easy to figure out how an "inappropriate" activation of what would otherwise be an immature, tolerogenic DC may lead to the break of peripheral tolerance. Multiple factors inducing DCs maturation, and rendering them more effective by enhancing antigen-processing machinery, display of major histocompatibility complex (MHC) molecules at their surface, expression of co-stimulatory molecules or secretion of cytokines and chemokines can convert a tolerogenic presentation of self-Ag by DCs into a presentation leading to an activation of circulating autoreactive T and B cells. This concept was elegantly demonstrated in a recent model where Influenza virus-induced dendritic cell maturation is associated with the induction of strong T-cell immunity to a co-administered, normally non-immunogenic protein [114].

Many autoimmune diseases can be triggered by microbial infections (reviewed in [115]). Two theories prevail to explain the causative link between microbial infection and autoimmunity. Autoimmunity arise because the immune system reacts against an epitope of the pathogen, which is similar to one of the host itself ("epitope mimicry") [116]; Autoimmunity arise because the infection provides an activation of the immune system which, prone to dysfunction, sooner or later mounts an autoimmune reaction ("bystander activation") [117]. Indeed, pathogenic autoimmunity can occur during the onset of an infection without any epitope mimicry, only due to a nonspecific inflammatory effect. One of the strong mechanisms involved in this nonspecific activation of the immune system stands in a "bystander activation" of the DCs. The functional differentiation of DCs may be induced by viruses, bacteria or parasites directly through a variety of receptors specifically recognizing microbial products [118] or indirectly by inducing the synthesis of pro-inflammatory cytokines such as type I IFN, tumor necrosis factor-a (TNF-a), interleukin 1 (IL-1). Host cell destruction and CD40/CD40 ligand signaling pathway provide further stimulus to activate DCs. For example, the combination of signaling through TLR9 and CD40, an association of two major DCs stimuli, which usually occurs in almost every bacterial infection, breaks the tolerance that mice have developed for a self-Ag [119]. In another model, when CD40L is over-expressed in the epidermis, LCs are chronically mature and systemic autoimmunity develops [120]. During infections, the triggering of autoimmunity is also favored by other factors such as epitope mimicry, host cell destruction and the release of large quantities of normally sequestered Ag, the effect of pro-inflammatory cytokines production on cells other than DCs, and a polyclonal lymphocyte activation *via* either a mitogen or a superantigen effect.

Direct stress, irradiation, tissue necrosis and in some settings, apoptotic cells may also activate DCs (reviewed in [121]). It is interesting to note that these factors

are usually considered to trigger in many clinical circumstances flares of autoimmune diseases.

The concept of an inappropriate activation of DCs in autoimmune diseases has been specially explored in SLE where the break in peripheral tolerance might be caused by to activation of mDCs in response to an excess of IFN-ab [122].

45.4.1.1 Systemic Lupus Erythematosus as an IFN-a Driven Disease

SLE is a prototype autoimmune disease characterized by a break of tolerance to nuclear components and profound alterations of the immune system [123–127]. Type I IFNs (IFN-ab) have gained considerable interest as candidates in the etiopathogenesis process of lupus (reviewed in [128]). A fraction of patients display detectable circulating IFN-a in serum that correlates with both disease activity and severity, as detected using Elisa or antiviral bioassays [129–131]. Moreover, some reports showed that a high proportion of mononuclear cells from SLE patients with IFN-a-positive or-negative sera expressed IFN-a inducible 2–5A synthetase and Mx proteins [132, 133]. Yet, microarray analysis of blood mononuclear cells demonstrates that the vast majority of patients we analyzed display an IFN signature [134, 135]. IFN-a treatment of patients with cancer or infectious diseases can induce the development of a broad spectrum of different auto-antibodies, sometimes associated with overt organ-specific and systemic autoimmune diseases and more specifically anti-dsDNA auto-antibodies and SLE. Those observations have pointed out the possible causative link between IFN-a and lupus [136, 137]. In patients who actually developed SLE, discontinuation of IFN-a treatment and prednisone administration led to remission of the disease [130, 136].

$CD14^+$ blood cells are usually immunologically quiescent monocytes, unable to mount the so-called mixed lymphocyte reaction (MLR). However, in SLE disease, these cells are able to induce the proliferation of alloreactive T cells, a property characteristic of DCs [122]. Indeed, incubation of healthy individuals' monocytes with serum from active SLE patients generates cells with certain characteristics of DCs, including their phenotype and their ability to induce an MLR. IFN-ab was known to activate immature mDCs *in vitro* [138–140]. Using neutralization experiments, IFN-a have been demonstrated to be the key SLE serum factor responsible for the differentiation of monocytes into DCs. Furthermore, normal serum spiked with recombinant IFN-a can also induce monocyte differentiation into DCs. SLE serum also contains other cytokines that may participate in the DC-induction effect. In particular, high levels of CD40 ligand are found [124, 141, 142]. These IFN-a driven DCs capture apoptotic cells and present their antigens to T cells [122]. Indeed, SLE may be considered as a problem in the processing of apoptotic cells by DCs [143–145]. Because IFN-a exert pleiotropic effects, their role as pathogenic effectors in SLE is likely to be mediated by other mechanisms including activation of T and B cells differentiation; imbalance between pro-and anti-inflammatory cytokines; induction of chemokines/chemokine receptors to promote the homing of immune cells into inflamed tissues and a direct pathogenic effect on target tissues.

45.4.2
Defective Downregulation of Activated Dendritic Cells

Most activated DCs quickly disappear after having activated lymphocytes [146, 147]. This downregulates the magnitude of T cells responses. *In vivo*, lifespan of activated DCs is highly regulated through the expression of Bcl-2 family members [148]. One can imagine that a failure in the downregulation of activated DCs may lead to an excess of stimulation and give rise to autoimmunity. This emerged with the discovery that suppressor of cytokine signaling-1 (SOCS-1) plays an essential role in the normal DC functions and suppression of systemic autoimmunity. $SOCS^{-/-}$ negatively regulates, not only the cytokine-signaling pathway, but also lipopolysaccharide (LPS)-induced activation. $SOCS1^{-/-}$ DCs are hyper-responsive to IFN-a and IL-4. $SOCS^{-/-}$ DCs induced B-cell expansion and autoreactive antibody production *in vivo*, an SLE like phenotype [149].

Such a same defect in the control of DCs activation has not yet been described in human autoimmune pathology. However similar mechanisms have been reported. An intrinsic protection from apoptosis by mutations in apoptosis controlling genes may lead to the persistence of activated DCs during an immune response whereas they should have disappeared from the secondary lymphoid organs. Those DCs could prime an autoimmune response simply by a long extended stimulation. In this manner, mutations in the caspase 10 gene were described in patients with type II autoimmune lymphoproliferative syndrome, a rare disease associated with autoimmune features such as autoimmune hemolytic anemia and thrombocytopenia [150, 151]. These patients DCs are protected from FAS ligand or TNF-related apoptosis-inducing ligand apoptosis and tend to accumulate in the T zone of lymph nodes [152].

Another example is found during the exogenous administration of GM-CSF. This well-known growth and survival factor for DC *in vivo* and *in vitro*, induces an accumulation of DCs in secondary lymphoid organs and might thus exacerbate pre-existing autoimmunity. For example, exacerbation of autoimmune thyroiditis, thrombocytopenia, arthritis or the development of cutaneous leukocytoclastic vasculitis, often in a dose-dependent manner, resolving after treatment discontinuation, have been described [153].

45.4.3
The Rise of "Autoimmune-prone" Dendritic Cell Subsets

Different DC subtypes, with different phenotypes and functions arise from separate developmental pathways, e.g. mDCs, pDCs, LCs and iDCs in human [9–11]. Besides, cytokines produced during an ongoing immune response skew the differentiation of monocytes into different DCs subsets generating, for example IL-4-DCs, IFN-a-DCs, TNF-a-DCs, TGF-b-DCs, IL-15-DCs or IL-10-DCs (reviewed in [21]) (Fig 45.2). Their development and functions are actually even further modulated by exogenous factors [154]. Each DC subset displays common as well as unique combination of enzymatic machinery, cell surface molecules and cytokines

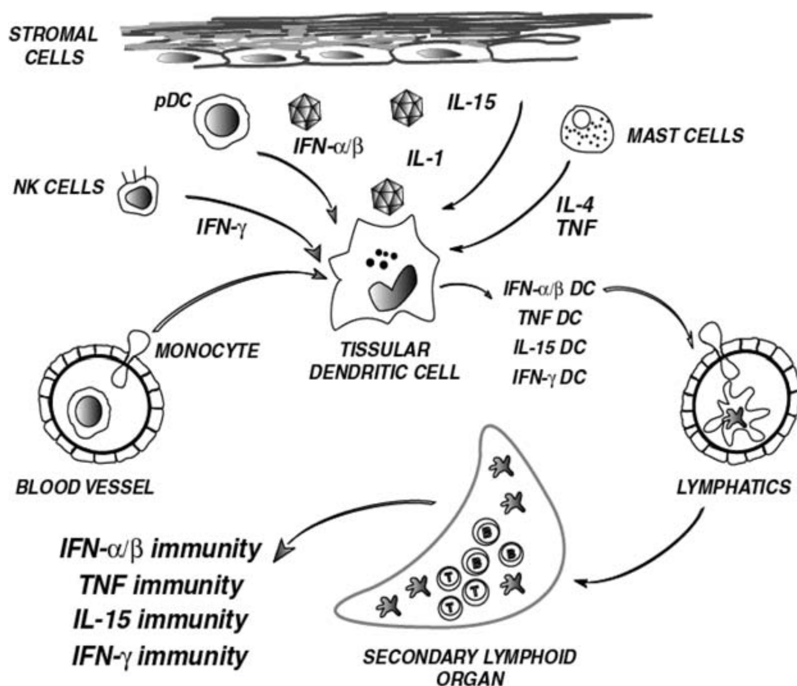

Fig. 45.2 Cytokine microenvironment determines differentiation/maturation of dendritic cells and ensuing immune responses. Pathogens can induce DC maturation directly via pattern recognition receptors on DCs or via triggering other cell types including stromal cells (epithelial cells, keratinocytes, and fibroblasts), mast cells, plasmacytoid DCs and NK cells to secrete different cytokines: IFN-α, TNF, IFN-γ, IL-4, IL-15, IL-1 etc. These cytokines activate DCs differentially yielding distinct subsets that induce distinct types of immune responses.

secretion and thus different functional properties [155]. For instance, a specific DC subset will be particularly efficient in priming self-specific T cells, whereas another one in inducing T cells which regulate or suppress other T cells, or again, another one in shifting a Th1 into a Th2 profile. Therefore, one can easily imagine that, during an immune response, the emergence or the lack of certain DC subsets can orchestrate a skew in the activation/differentiation of T cells clones which will eventually lead to autoimmunity.

An example can be *found in the processing of Ag by DCs.* IFN and TNF-a induce the expression of new proteasome subunits altering their pattern of peptidase activities. These cytokine-induced replacements of proteasome subunits are believed to favor the production of peptide ligands of MHC class I molecules for the stimulation of cytotoxic T cells, sometimes being crucial to mount a reaction against several epitopes [156]. IL-6 has also been found altering the processing of Ag in DCs by modifying the acidification in the endosome. Thus, DCs treated with IL-6 now process, present and activate T cells *in vitro* and *in vivo* against previously cryptic

determinants [157]. Different panels of enzymes induced under the influence of different cytokines are likely to degrade a given Ag into a different peptide repertoire. Thus, distinct DCs subsets with distinct proteolytic enzymes will generate distinct repertoire of peptides leading to a distinct set of MHC peptide complexes, and, thus, a distinct Ag-specific T-cell repertoire as discussed later. That may then explain the causative link between cytokines such as IFN, TNF-a or IL-6 and autoimmune disease. However, whether IFN, TNF-a or IL-6 treated DCs can process autoAg so that several previously ignored epitopes like cryptic or subdominant self epitope may now be efficiently presented to nontolerized T cells still remain to be studied in human DCs.

Another example may be found in the *control of T-cell differentiation by DCs*. The full activation of a self-reactive T-cell clone does not necessarily include a pathogenic outcome. Indeed, the same T-cell clone can be driven toward different profiles of cytokine/chemokine secretion, migration properties and effector functions. Some of those profiles are detrimental, whereas other profiles are protective when considering the autoimmune reaction outcome. The best example of this concept stands in the *Th1/Th2 paradigm*. Th1/Th2 imbalances are often critical in autoimmunity for the progression or remission of the disease. With few exceptions, Th1 response is often deleterious, while the shift to a Th2 response is protective (reviewed in [158]). DCs may regulate the Th1/Th2 balance [159] using various mechanisms, e.g. the nature of the DC subset itself, the nature of the pathogen products, cytokines and prostaglandins present in the microenvironment and the Ag dose [160–164]. The main feature of this control is its plasticity. For example, in one hand, if human pDCs generated with IL-3 and CD40L preferentially activate Th2 or CD8 T regulatory cells, in the other hand, they elicit a Th1 response when activated with influenza virus and CD40L [165–167]. By shaping the type of T-cell responses, DCs can then give rise to a Th1/Th2 imbalance that will eventually influence the outcome of the disease [168–170]. For example, intraepithelial DCs in atopic asthma selectively activate Th2 lymphocytes, whereas dermal DCs in psoriasis induce Th1 lymphocytes [171, 172]. As a therapeutic approach, one can imagine the use of specific DC subset to redirect the Th1/Th2 imbalance during the immune response [173].

Other examples may be found *outside the Th1/Th2 paradigm*. In a non-exhaustive list, we can mention here that: (1) TNF-a-DCs protect from EAE and collagen-induced arthritis [174]; (2) the modulation of DCs maturation by IL-10 potentiates the function of IDO, an enzyme critically involved in regulatory mechanisms [175]; (3) pDCs tolerize T cells under certain circumstances [167]. During an ongoing inflammation, a lack of these tolerogenic subsets of DCs may drive the immune response into a dangerous autoimmune reaction. On the opposite, IFN-a-DCs present in SLE patients may, in a very efficient manner, activate cytotoxic CD8 T cells [176].

However, if the activation of autoreactive T and B lymphocytes constitute a mandatory prerequisite, this is not enough to bring overt autoimmune diseases with ongoing tissue destruction. Indeed, subclinical autoimmune responses, such as isolated autoantibodies, can frequently be detected in healthy individuals, usually among family members of sick patients [177, 178]. It is also important here to men-

tion that auto-antibodies may be present many years before the onset of the autoimmune disease as reported in SLE and RA [179, 180]. In an animal model, the initial priming of autoreactive cells occurred at the time of infection but without causing clinical disease. Later, these T cells were activated by appropriate nonspecific stimuli causing the disease to occur [181]. Thus, factors perpetuating autoreactive response and leading to disease expression i.e. tissue destruction are perhaps more important than the initial break of tolerance. We [182] and others have recently shown in animals focusing on lupus that if subclinical autoimmune response can be induced by either mature myeloid DCs or by prolonged systemic expression of IFN-a, other susceptibility factors have to be present to induce long-lasting autoimmunity and clinical expression of disease [183]. The susceptibility factors leading to tissue injury may involve the innate immune response. In that case, a mobilization of an adaptive immune response leading to autoimmune priming will result in genetically predisposed individuals, in an *unabated activation of the innate response* leading to the destruction of the target tissue [184]. However, in many instances, *sustained activation* of autoreactive lymphocytes is still required for the development of autoimmune diseases [185]. This could also be provided by DCs.

45.5
Dendritic Cells Migrate into Inflammatory Sites and Maintain a Vicious Circle

In peripheral tissues, DCs are considered to be immature/steady state and are poor T-cell stimulator. They uptake self-Ag or innocuous-exogenous Ag and after migrating through the lymphatics, they consequently induce peripheral tolerance (see above).

In many human autoimmune diseases, DCs are found infiltrating the target tissue itself surrounded by T and B lymphocytes, with, sometimes, *de novo* formation of organized lymphoid structures. Different subsets of DCs have been identified: pDCs, intDCs, and LCs. DCs are thus found infiltrating osteoarthritic synovial tissues in rheumatoid arthritis (RA), the thyroid gland in Hashimoto's thyroiditis and Graves' disease, the skin in psoriasis and SLE, labial salivary glands in Sjøgren's syndrome, the cerebral spinal fluid in multiple sclerosis, the pancreas in IDDM, arterial walls in giant cell arteritis, or the liver in autoimmune hepatitis and primary biliary cirrhosis [186–194]. With a few exceptions [195], DCs in the inflammatory site display an activated phenotype and have a high potency to stimulate lymphocytes. For instance, the proliferative response of T cells induced by DCs isolated from diseased tissues is higher than that elicited by blood DCs or normal tissue DCs [196]. It is crucial here not to forget that in the target tissue, DCs are not isolated but take part in a more global recruitment of inflammatory cells such as T cells, B cells, macrophages, and granulocytes. It is also important to mention that the infiltration of DCs in the target tissue is a nonspecific feature of inflammation, and has also been described in many other inflammatory conditions than autoimmunity [193, 197, 198].

In some autoimmune diseases, a prolonged presentation of self antigens by DCs may be pivotal for the development of destructive lesions. One mouse model stresses the requirement of repeated administration of Ag loaded DCs to lead to full-blown autoimmune disease [185]. In another SLE mouse model, interruption in the administration of self-Ag loaded DCs has been rapidly followed by a decrease in the anti-dsDNA auto-antibodies titers [199]. This extended presentation of self Ag could directly take place in the target tissue itself. There, after a first autoimmune injury, excessively present or recruited DCs will perpetuate inflammation/destruction through a vicious circle: self-Ag capture, presentation to lymphocytes and subsequent activation of effectors and tissue destruction. DCs are recruited in excess into the target tissue, attracted by locally secreted chemokines [200]. Persistent inflammation and destruction of the target organ may also favor the persistence of autoimmunity by increasing the amount of self Ag delivered, and by generating bystander activation of DCs and/or self-reactive lymphocytes. DCs may, directly inside the target tissue, activate memory autoreactive lymphocytes or, after migration into the draining lymph node, repetitively activate naïve autoreactive T and B cells.

Whether DCs only play a passive role in the sustaining activation of tissue inflammation or if they are the primary cause of the inappropriate persistence of the autoimmune response is still to be defined. In fact, in a chronic inflammation, several other factors may be needed to perpetuate this reaction such as a poor clearance of the Ag [201–203], the persistence of a chronic infection, a lower threshold of stimulation necessary to activate lymphocytes or the excessive recruitment of inflammatory cell through chemokine secretion [126]. However, taken together, the presence of DCs in the target tissue might be crucial to maintain a persistent inflammation and tissue destruction.

In a therapeutic approach, i.e. after the beginning of autoimmune tissue destruction, it would be useful to define whether one can stop this process by depleting DCs or by blocking their harmful function.

45.6
Dendritic Cells: Failure to Maintain Peripheral Tolerance

Impaired function of DCs may impair the maintenance of peripheral tolerance. Although being almost totally speculative, the mechanisms by which impaired function and or maturation of DCs lead to autoimmunity may rely in their incapacity to delete autoreactive T cells or to induce regulatory T cells. So far, impairment of DC function has only been approached in the blood samples of patients by studying DCs, directly or after *in vitro* cultures of their precursors/progenitors. The analysis have been done by phenotyping them, and by measuring their ability to be activated, to secrete cytokines and to activate autologous or allogeneic lymphocytes. And so, defective abnormalities in DCs functions have been described in three kinds of diseases: (i) diseases which associate susceptibility to infection and autoimmunity

like the Wiskott–Aldrich syndrome (WAS) or the common variable immunodeficiency (CVID), (ii) IDDM, (iii) classical autoimmune diseases such as SLE, RA, Sjøgren's syndrome and multiple sclerosis.

Concerning WAS patients, an inherited disease in which individuals suffer from infection, eczema and autoimmunity, DCs have a normal surface phenotype, but they display dysmotility and aberrant cell trafficking *in vivo*. Those deficiencies certainly affect DCs migration and maturation *in vivo* [204–206]. In CVID, a disease characterized by hypogammaglobulinemia, defects in T-cell functions and autoimmune features, DCs display severely perturbed differentiation, maturation, and function. They also express markedly reduced levels of co-stimulatory molecules inducing weak proliferation of allogeneic T cells and produce low amounts of IL-12 [207].

In IDDM, a decreased maturation of DCs derived from blood monocytes with impaired capacity to stimulate autologous and allogeneic T cells has been reported among patients, and even more interestingly among relatives at risk for insulin-dependent diabetes [208, 209]. The importance of this feature in the pathogenesis of IDDM has even been further demonstrated in the NOD mouse, a mouse model of IDDM. Indeed, the passive transfer of DCs into prediabetic animals prevents the onset of the disease presumably by correcting an intrinsic regulatory defect in these mice [210].

Impairment in the numbers of blood DCs, in their phenotype and their function have also been described in many other autoimmune diseases such as SLE [211, 212], Sjøgren's syndrome and RA [213], although not in MS [214]. Whether this impairment contributes to the pathogenesis still remain elusive. They may only be left-over DCs or progenitors/precursors after an activation or a selective recruitment into target tissues, rather than a primary or secondary defect in DCs ontogenesis. In this regard, it is important to remember here that in many of these autoimmune diseases, DCs are actually present in large excess in the target tissues where they display an activated phenotype and an increased stimulatory capacity (see above).

45.7
A Special Role for Plasmacytoid Dendritic Cells in Systemic Lupus Erythemathosus

DC numbers are reduced in SLE blood. Myeloid DCs (mDCs), characterized as $CD14^{-}CD11^{hi}HLA\text{-}DR^{+}$ cells, are reduced by about 30% in the blood of SLE patients, and plasmacytoid DCs (pDCs), characterized as $CD123^{hi}HLA^{-}DR^{lo}$, and Lin^{neg}, are reduced by as much as 70% [122, 211, 212]. This paradoxical finding may be explained by recruitment of pDCs to sites of target tissues, e.g. cutaneous lesions in SLE [215]. The way pDCs in SLE may be involved in the pathogenesis of SLE is just starting to be elucidated. pDCs may be the main type I IFN-a producer, and thus consequently induce the overproduction of plasma cells observed in this disease.

45.7.1
Plasmacytoid Dendritic Cells as the Main Producer of Type I IFN in Systemic Lupus Erythemathosus

It has earlier been demonstrated that plasmacytoid DCs (pDCs) resting in lymph nodes or circulating in blood can secrete large amounts of IFN-ab upon exposure to viruses as well as bacterial DNA [46–49]. The origin of IFN-a, and mechanisms involved in IFN-a production in SLE, still remain obscure. Knowing the deep alterations of pDCs in human SLE, the assumption that pDCs are the main producer of IFN-a in human SLE can consequently be made [128, 216, 217]. A recent report involving studies in mouse model seems to confirm that pDCs are responsible for the IFN-a over-expression in SLE [218]. It has not been established whether IFN-a is turned on in lupus because of a primary defect such as hyperproduction or hyper-responsiveness to stimuli, or due to a secondary abnormality, such as a microbial infection, or increased level of anti-dsDNA/DNA immune complexes and/or apoptotic bodies. In fact, anti-dsDNA/DNA immune complexes and/or apoptotic bodies have been shown to induce IFN-a production by pDCs and might therefore constitute an amplification loop in disease [219–221]. pDCs are probably not the only source of type I IFN-a secretion. In fact a second amplification loop of IFN-a secretion has also been described recently: IFN-ab triggers TLR7 expression on monocytes and DCs. As a result, it has been demonstrated that virus-derived single stranded RNA, a TLR7 agonist, can therefore induce these cells to produce IFN-ab [222–224].

45.7.2
Plasmacytoid Dendritic Cells Induce Plasma Cell Differentiation

Alteration of the B cells compartment, increased generation of plasma cells and production of auto-antibodies are well known features of both human and mouse SLE [126]. IFN-ab is an excellent adjuvant for humoral immunity [225]. Given the central role of IFN-ab in SLE, the question arises as to if IFN-ab might be responsible for the increased frequency of plasma cells in this disease [34]. Activated B cells exposed to influenza virus-triggered pDCs differentiate into Ig-secreting plasma cells in both IFN-ab- and IL-6-dependent manners [34]. Interestingly enough, the plasma cells generated under these conditions convey very high levels of CD38, similar to the level of expression on plasma cell found in excess in SLE blood [226, 227]. Plasma cell differentiation may also arise through myeloid DCs activation by IFN-ab. Indeed, myeloid DCs can induce B cells growth and differentiation. [228–232]. Several molecules have been shown to be involved in this process, including IL-12, IL-6 [10], and, more recently, BAFF/Blys [233–237], a molecule upregulated *in vivo* by IFN-a. Its over-expression in mice results in SLE-like and Sjøgren-like phenotype [233, 238–240]. Furthermore, increased levels of Blys are found in SLE patients [241–243].

45.8 Dendritic Cells Fail to Delete Developing Autoreactive T Cells

An important role for central tolerance mechanisms has been reemphasized by the discovery that a recessively inherited disorder called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune polyendocrinopathy syndrome type 1 (APS1), a disease combining several autoimmune endocrine disease was due to a mutation in the gene encoding for the autoimmune regulator (AIRE/aire) [244, 245]. This gene encodes a protein which has evocative features of a transcription factor and is assumed to control the promiscuous transcription in the thymus of peripheral tissue-specific proteins. Their presentation by thymic epithelial cells or DCs within the thymus may lead to the induction of T-cell tolerance against tissue specific self-Ag [246]. However, experimental findings in mouse argue against a required role for aire expression in DCs, even though they express the molecule [246]. However the possibility that a lack of central tolerance may be the cause of certain autoimmune disease is now established in human pathology. DCs may play a crucial role which is still to be discovered.

45.9 Autoimmunity Through Cytokine-induced Dendritic Cell Activation

As already discussed above, cytokines are key players in autoimmunity. Type I interferons (IFN-$\alpha\beta$) play a role in SLE. TNF plays a critical role in rheumatoid arthritis [247] and psoriasis [248] and TNF antagonists have proven to be efficient in therapy of these diseases [249]. However, these antagonists may lead to clinical complications such as reactivation of tuberculosis [250] and reversible SLE (reviewed in [251, 252]). Conversely, anti-TNF therapy is not effective in patients with SLE and may in fact worsen clinical symptoms of this disease. These clinical observations suggest that TNF might regulate IFN-α production. Indeed, TNF antagonists enhance the production of IFN-$\alpha\beta$ by pDCs exposed *in vitro* to viruses. Furthermore, treatment of patients suffering from systemic onset juvenile idiopathic arthritis with anti-TNF induces overexpression of IFN-α-regulated genes in blood leukocytes [253]. These results may thus provide a mechanistic explanation for the increased anti-dsDNA antibodies and lupus like syndrome in a fraction of patients treated with TNF antagonists. Conversely, TNF inhibits IFN-α release by pDCs exposed to Influenza virus, and completely blocks pDCs generation from $CD34^+$ hematopoietic progenitors [253]. Thus, high levels of soluble TNF receptors in SLE [212, 254, 255] may block endogenous TNF. The TNF-mediated down-regulation of the type I interferon pathway could also explain earlier observations in the mouse lupus model NZB/W, which bear a genetic deficiency of TNF [256]. Consequently, these mice benefit from replacement therapy with recombinant TNF [257].

On the other side of the spectrum, there is evidence that IFN-$\alpha\beta$ may regulate TNF. For example, TNF is implicated in the pathogenesis of multiple sclerosis

[258, 259] and is involved in EAE, an experimental model for multiple sclerosis. IFN-β knockout mice seem more susceptible to EAE than the wild type [260] and administration of IFN-β to mice with EAE inhibits disease progression [261]. In humans, IFN-β can inhibit *in vitro* TNF production by microglia, either directly or by attenuating the ability of T cells to trigger TNF secretion by microglial cells [262].

Taken together our data open a new way to look at the control of autoreactive immune responses which have been challenging to integrate within the Th1/Th2 paradigm [159, 263]. We therefore propose to integrate two antagonistic paths i.e. the IFN path and the TNF path. These paths lead to contrasting immune responses, which might yield opposite pathologies, i.e. lupus and arthritis.

45.10
Different Cytokines Generate Different Dendritic Cells that may lead to Different Autoimmune Syndromes

In response to microbial infection, cells from the invaded tissue secrete different cytokines. As discussed above, these cytokines will induce attracted monocytes to differentiate into different DCs, for example IL4-DCs [264–266], IFN-DCs [122, 138–140], TNF-DCs [267], IL15-DCs and IL1-DCs (Fig 45.2). Each DC subset displays common as well as unique biological functions determined by a unique combination of cell surface molecules and cytokines. For example, distinct DC subsets with distinct proteolytic enzymes will generate distinct repertoire of peptides leading to a distinct set of MHC peptide complexes and thus, a distinct antigen-specific T-cell repertoire. This concept can be illustrated by TNF and IFN-αβ driven differentiation of distinct types of DCs. Thus, IFN-DCs will present nuclear antigens eventually leading to generation of anti-dsDNA antibodies typical of SLE. In contrast, TNF-DCs will present Fc fragment of immunoglobulins eventually leading to generation of rheumatoid factor typical of rheumatoid arthritis.

We therefore propose a simple view of dendritic cells and ensuing immunity as a wind rose which is composed of TNF/IFN-α and IL-4/IFN-γ (Fig. 45.3). There, immunity is viewed as a dynamic system driven by two sets of opposite vectors, i.e. TNF and IFN-α and IL-4 and IFN-γγ. When vectors are equal, protective immunity can commence. However, when one of the vectors prevails beyond a certain threshold, immunopathology such as autoimmunity, allergy or inflammation occurs. We have recently extended this model to other cytokines by demonstrating the role of IL-1 in Systemic onset juvenile idiopathic arthritis (SoJIA) [268]. SoJIA encompasses ~10% of cases of arthritis that begin in childhood. The disease is unique in terms of clinical manifestations, severity of joint involvement and lack of response to TNF blockade. We have now shown that serum from SoJIA patients induces the transcription of innate immunity genes including Interleukin-1 (IL-1) in healthy peripheral blood mononuclear cells (PBMCs). Upon activation, SoJIA PBMCs release large amounts of IL-1beta. Furthermore, administration of recombinant IL-1 receptor antagonist to 14 SoJIA patients refractory to other therapies induced complete remission in 12/14 patients. Thus, IL-1 is a major mediator of the

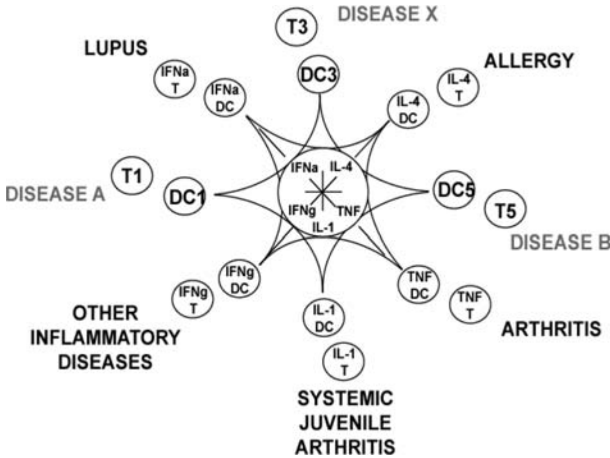

Fig. 45.3 Cytokine microenvironment determines differentiation/maturation of dendritic cells and ensuing immune pathology. A wind rose of immune homeostasis and immune pathology. Each set of autoimmune diseases displays a unique lymphocyte alteration which can be traced to a specific DC induced by a specific cytokine. Disease results from alteration in cytokine equilibrium that permits immune homeostasis as well as protective immunity.

inflammatory cascade underlying SoJIA and represents a target for therapy in this disease.

45.11 Concluding Remarks

DCs are the critical decision-making cells in the immune response. A large amount of data coming directly from studies of human diseases confirms the strong involvement of DCs in the priming and the persistence of autoimmunity. Current treatments employ Ag-nonspecific immune suppressants that globally block lymphocyte activation and cytokine production. To define new therapeutic strategies in autoimmune diseases, it is crucial to understand the mechanisms by which autoimmune responses are generated, how the DCs drive the immune pathology and how they might be stopped.

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XVI
Transplantation

46
Role of Dendritic Cells in Graft Rejection and Graft-versus-host Disease

Véronique Flamand and Michel Goldman

46.1
Alloantigen Presentation in Organ Transplantation

46.1.1
Pathways of Alloantigen Recognition

Molecules encoded by the major histocompatibility complex (MHC) are the dominant antigens causing acute or chronic allograft rejection. Class I and class II MHC molecules are most important because of the extraordinary polymorphism they exhibit. MHC class I molecules are expressed on all nuclear cells whereas MHC class II molecules are expressed on restricted cell types such as macrophages, dendritic cells, B cells, endothelial cells and activated human T cells. MHC class I alloantigens are mainly recognized by the T-cell receptor of $CD8^+$ T lymphocytes, whereas MHC class II alloantigens are recognized by the T-cell receptor of $CD4^+$ T lymphocytes. Minor histocompatibility antigens, consisting in peptides of self-proteins which are presented in an immunogenic form by MHC molecules, may also trigger acute or chronic allograft rejection.

46.1.1.1 The Direct and Indirect Pathways

The allo-immune responses against foreign MHC antigens are induced either by intact allogeneic MHC molecules (the direct pathway of allorecognition) or by peptides derived from polymorphic sequences of allogeneic MHC molecules presented by self-MHC molecules (the indirect pathway of allorecognition) (Fig. 46.1). In addition to alloantigen recognition, T-cell activation requires the provision of co-

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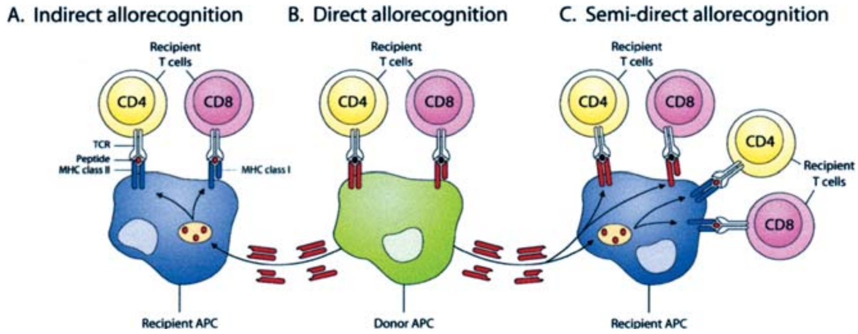

Fig. 46.1 Pathways of allorecognition. In the indirect pathway (A), alloantigens are either shed from donor cell surface or are taken up as dying or apoptotic allogeneic cells by recipient antigen-presenting cells (APC). Peptides (red circle) derived from allogeneic MHC class I or class II molecules are re-presented on the self-MHC class II or class I molecules of recipient APC. In the direct pathway (B), recipient $CD4^+$ and $CD8^+$ T cells recognize respectively donor intact allogeneic MHC class II or class I molecules bound to peptides at the surface of donor APC. In the semi-direct pathway (C), recipient APC would present to recipient T cells intact MHC class I and class II molecules transferred from donor APC simultaneously with donor MHC peptides to $CD4^+$ and $CD8^+$ T cells with indirect allospecificity.

stimulatory signals delivered by antigen-presenting cells (APC). In organ transplantation, activation of the direct allorecognition pathway depends on graft-resident, hematopoietic, professional APC such as dendritic cells (DC). Several types of cells have antigen-presenting capability, including skin-specific DC, i.e. Langerhans cells (LC), liver-specific macrophages (i.e. Küppfer cells) or activated B cells. It was first thought that cognate interactions between naïve T cells and nonprofessional APC such as resting nonhematopoietic endothelial cells would lead exclusively to tolerance [1]. However, it was reported that donor endothelial cells may also represent potent APC for alloreactive T cells as was demonstrated for cardiac transplant acute rejection mediated by alloreactive $CD8^+$ T cells which directly recognize donor MHC class I on graft endothelium [2].

On the other hand, the indirect alloantigen presentation pathway depends on recipient DC presenting donor-derived peptides. The nature of DC involved in direct presentation compared with indirect is supposed to be different, as the former will be tissue-resident DC while those from the recipient will derive from blood or be associated with lymphoid tissues. Endothelial cells could also play a role in the indirect pathway of alloantigen recognition. Inflamed host endothelium that repopulates donor allografts can indeed process exogenous donor antigens and present the relevant peptides to recipient $CD8^+$ T cells in the context of recipient MHC class I. The $CD8^+$ T cells that recognized the crosspresented antigen on endothelial cells were demonstrated to be responsible for skin allograft rejection [3].

The high frequency of T cells with direct allospecificity and the low frequency of T cells with indirect allospecificity in the normal T-cell repertoire have led to the suggestion that the direct alloresponse dominates the early phase after transplantation, while the indirect pathway plays a major role in the later phase of allo-immune responses when donor-type APC disappeared from the transplanted organ. This implies that the direct pathway could play a dominant role in acute rejection and is unlikely to contribute to chronic rejection. Indeed, in renal and cardiac transplanted patients, it was demonstrated that the frequency of T cells with direct anti-donor allospecificity declines with time [4, 5]. However, the indirect pathway is likely to be permanently active due to the traffic of recipient APC through the graft. Indeed, T cells with indirect anti-donor specificity increase in frequency in patients with late graft failure.

It is important to realize that the use of the terms "direct" and "indirect" presentation sometimes might be confusing. First, direct presentation does not necessarily imply an absence of antigen processing, as peptides of donor MHC class I molecules are presented by donor class II antigens or even other donor class I antigens. Second, when donor and recipients share certain MHC antigens, peptides presented by indirect and direct pathway may be identical.

46.1.1.2 **The Semi-direct Pathway**

Whether the direct or the indirect pathway necessarily involves distinct APC is a matter of debate. Indeed, Lee et al. [6] reported first that helper $CD4^+$ T cells with indirect anti-donor allospecificity could amplify an effector $CD8^+$ T cell response with direct anti-donor allospecificity. Furthermore, Wise et al. [7] identify a mode of "linked suppression" where $CD4^+$ regulatory T cells activated through an indirect pathway could regulate effector $CD8^+$ T cells with direct specificity for donor alloantigens, as long as the grafts cells carry both alloantigens. A new pathway of alloantigen presentation, called semi-direct pathway, has recently been proposed by Lechler et al. [8] who demonstrated *in vitro* the transfer of intact MHC class I and class II molecules from murine allogeneic recipient DC or endothelial cells to donor DC (Fig. 46.1). Such acquisition of recipient MHC molecules was visualized *in vivo* and evidence was provided that these acquired MHC complexes were efficiently presented to alloreactive T cells. Recipient DC would acquire and present intact donor MHC class I and class II directly to alloreactive T cells and simultaneously present processed donor MHC peptides indirectly to recipient alloreactive T cells.

Another source of intact allogeneic MHC molecules comes from exosomes. Exosomes are membrane vesicles that result from the fusion of late multivesicular endosomes with plasma cell membrane and that are released by hematopoietic cells. Depending on their origin, exosomes are enriched in different molecules. DC-derived exosomes contain functional MHC class I and class II antigens and T-cell co-stimulatory molecules. Those APC-derived exosomes can be considered as a source of intact MHC class I and class II molecules that can be acquired by other DC *in vivo* and therefore prime $CD8^+$ and $CD4^+$ T-cell responses [9, 10]. Exo-

somes could therefore be used for immunization. It is interesting to note that, depending on the context of their administration, allogeneic exosomes may induce tolerance instead of immunity. It was indeed demonstrated that treatment of heart allograft recipients with intravenously administered donor rat DC-derived exosomes delayed acute allograft rejection [11].

46.1.2
Sites of Alloantigen Presentation

It was first observed by Barker et al. that draining lymph nodes are the primary sites of sensitization for skin graft rejection [12, 13]. Donor-derived DC have been recognized as the principal instigators of allograft rejection because of their migratory properties. It was indeed demonstrated that donor DC from cardiac allografts were able to migrate to recipient spleen after transplantation, implying that direct alloresponses could be initiated in lymphoid tissue [14]. Lakkis et al. later demonstrated that cardiac allografts were immunologically "ignored" when recipients lack secondary lymphoid organs, indicating that, similar to the immune response to a pathogen and a self-antigen, allo-immune responses responsible for allograft rejection are initiated in lymphoid tissue [15].

Changes in the location of both donor and recipient APC take place almost immediately after transplantation. In the early phase of post-transplantation the graft contains mostly donor DC as well as few recipient's DC. Donor APC begin to migrate from the graft to the recipient lymphoid compartments first in the draining lymph nodes and then in the spleen of the recipient [16]. Simultaneously, bone-marrow-derived APC from the recipient begin entering the graft and constantly traffic through the graft and internalize, process and present soluble alloantigens. So during the early phase of the anti-donor immune response post-transplantation, both donor DC and recipient DC can stimulate the direct and the indirect pathways of allorecognition in the recipient's lymph nodes. In the late phase of post-transplantation, recipient's DC have gradually replaced the donor APC. Donor DC die in the lymph nodes and their MHC products can be processed by the recipient DC. The indirect anti-donor alloresponse would therefore last as long as living graft cells persist.

One recent study suggests that secondary lymphoid organs are important but not absolutely required for allograft responses [17]. There is also evidence suggesting that activation of alloreactive T cells can occur within the grafts, especially when they are primarily vascularized and contain donor endothelial cells [18]. MHC alloantigens expressed on the resident cells of the graft without migratory capacities such as endothelial cells, parenchymal cells and epithelial cells most probably contribute to local activation of circulating alloreactive T cells.

46.1.3
Factors Inducing Dendritic Cell Maturation and Migration

In the peripheral tissues, donor and recipient immature DC have high capacities of antigen endocytosis and phagocytosis, high expression of intracellular MHC class II molecules and a low expression of co-stimulatory molecules such as CD80, CD86 and CD40. In order to prime alloreactive naïve T cells, these immature sentinels must convert into mature immunostimulatory DC. Several factors contribute to DC maturation in the transplantation setting.

46.1.3.1 Ischemia/reperfusion Injury

Ischemia/reperfusion injury is a major determinant of organ dysfunction in the early post-transplant period. Organ ischemia was shown to modify the immunogenicity of liver allografts through upregulated expression of adhesion and MHC class II molecules [19]. Furthermore, ischemia/reperfusion and the transplantation procedure itself were reported to be sufficient to induce recruitment of recipient MHC class II-positive DC into kidney allografts [20]. Likewise, we demonstrated that hepatic ischemia/reperfusion evokes DC recruitment and maturation [21].

During organ transplantation, factors that favor DC maturation include oxidative cellular stress, necrosis and apoptosis induced by allograft ischemia/reperfusion. First, graft cells apoptosis was shown to be a key event in the inflammatory cascade elicited by ischemia/reperfusion injury. At the time of reperfusion, cell apoptosis is induced by reactive oxygen species, cellular ATP depletion, upregulation of death ligands like TNF-α and CD95L, and complement activation products [22, 23]. Immature DC may internalize apoptotic cells of both early and late phases [24]. DC that had taken up apoptotic cells in the early phase acquired a non-fully mature DC phenotype, expressing low MHC class II complex, co-stimulatory molecule CD40, and had a low capacity to stimulate allogeneic $CD4^+$ T cell proliferation. DC that had taken up apoptotic cells in the late phase acquired a mature DC phenotype with enhanced T cell stimulatory capacity [25]. Reactive oxygen intermediates may also directly act on DC to promote their maturation [26].

Second, necrotic cell death may lead to the release of heat shock proteins (HSP) that bind to CD91 and elicit NF-κB dependent maturation of dendritic cells [27–29]. Oxidative cellular stress, necrosis and apoptosis induce the release of inflammatory cytokines such as TNF-α and IL-1 by endothelial and parenchymal cells. TNF-α and IL-1 are central mediators of inflammatory responses associated with ischemia/reperfusion. They could first dictate the maturation of resident donor DC and furthermore induce the expression of adhesion molecules on vascular endothelium cells including β2 integrins, E- [30], P- and L-selectins [31, 32], and ICAM-1, as well as chemokine production including the neutrophil-attractants CXCL8, CXCL1 (Gro-α) and CXCL2 (MIP-2) that would lead to a subsequent graft influx of polymorphonuclear neutrophils. For example, graft ischemia/reperfusion injury is attenuated when neutrophils recruitment is decreased through Gro-α and MIP-2 inhibition [33].

Finally, the movement of donor or recipient's maturing DC depends on chemokine gradients [34]. Immature DC express CCR6 and respond to MIP-3α, whereas maturing DC downregulate expression of CCR6, upregulate CCR7 and respond to MIP-3β [35]. This regulation of chemokine receptors allows immature DC to be recruited to inflammatory sites and to leave this site after alloantigen capture to reach secondary lymphoid organs. Activated neutrophils may release several factors that would further activate intragraft DC or eventually attract recipient's DC ([34], reviewed by [36]). During microbial infection, it was indeed demonstrated that neutrophils produce CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CCL20 (MIP-3α) which chemoattract immature DC [37]. Alpha-defensins, contained in azurophil granules, also form chemotactic gradients for immature DC [38]. Likewise, properdin stored in secondary neutrophil granules, facilitates the formation of C5a which promotes recruitment of immature DC [39].

A new pathway of immature DC recruitment has been identified. Chemerin is a novel chemotactic agent identified as the natural ligand of ChemR23, a receptor structurally related to receptors for chemokines and other chemoattractant molecules and expressed in activated macrophages and monocyte-derived immature DC [40]. Chemerin is synthesized as a secreted precursor, prochemerin, which is poorly active and requires a proteolytic activation that could be induced during blood coagulation. *Ex vivo*, it was recently shown that chemerin attracts plasmacytoid DC but not myeloid DC [41] even if both cell types express ChemR23. Furthermore, in lupus erythematosis skin lesions, chemerin is produced by endothelial cells and is attracting selectively plasmacytoid DC [42].

46.1.3.2 **Links between Innate and Adaptive Immunity during Allograft Rejection**

The recently discovered Toll-like receptors (TLR) expressed on APC have been first identified as critical receptors for innate immune recognition of microbial pathogens [43]. Thereafter, TLR signaling was shown to contribute to inflammatory responses in other settings including transplantation. In a HY-mismatch skin allograft model in mice with targeted mutation of MyD88, a common TLR adaptor molecule, Goldstein et al. demonstrated that allograft rejection is critically dependent on signaling via MyD88 [44]. It was therefore hypothesized that TLR signaling on either donor or recipient DC during organ transplantation would lead to DC maturation and migration [45]. Several studies have demonstrated that endogenous ligands, including HSPs [46], heparan sulfate [47], oligomers of hyaluronan [48], and fibrinogen, and the content of necrotic cells can deliver signals via TLR4. Those ligands may be expressed during allograft rejection. Indeed, Palmer et al. provided evidence that lung transplant recipients with TLR4 points mutations show a delayed and reduced incidence of acute rejection [49]. In models of skin or cardiac allografts across a full MHC mismatch, DC allo-immune priming and IFN-γ production by alloreactive T-cells were then demonstrated to be significantly reduced in the absence of MyD88 on both recipient- and donor-type cells [50].

46.2 Alloantigen Presentation during Graft-versus-host Disease

46.2.1 Dual Impact of Alloreactive T Cells during Graft-versus-host Disease

Allogeneic bone marrow transplantation is the major approach to treat leukemia and haematological genetic disorders. Hematopoietic cell transplantation is more complex than organ transplantation since the administration of a donor graft that contains mature T cells to a conditioned immuno-incompetent recipient is associated with a risk of a graft-versus-host disease (GVHD) (reviewed by [51]) (Fig. 46.2). Those T cells primarily target specific organs, such as skin, liver, and intestine. The alloreactive donor T cells also have a beneficial role since they favor the engraftment and also significantly contribute to the anti-leukemia reactivity via the graft-versus-leukemia (GVL) reaction. GVHD can be avoided by removing donor T cells from the inoculum, but this increases the risk for tumor relapse [52]. Interestingly, natural $CD4^{+}CD25^{+}$ regulatory T cells might prevent GVHD while maintaining the GVL effect [53].

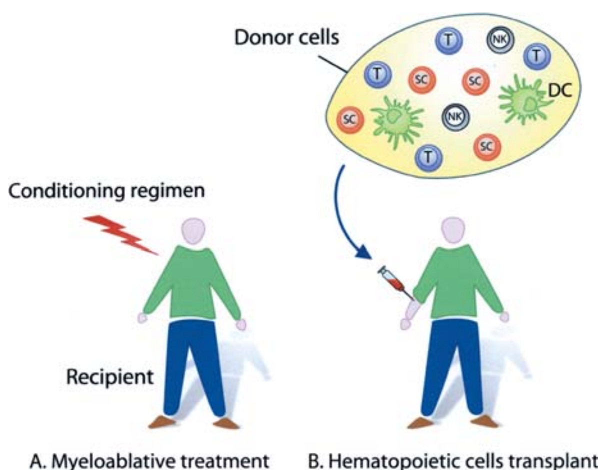

Fig. 46.2 Hematopoietic cells transplantation. Haematopoietic cell transplantation procedures consist of a preparatory conditioning regimen followed by the transplantation itself. (A) The cancer patient is treated with chemotherapy and irradiation to kill tumor cells and ablate the bone marrow. (B) The patient is infused with a preparation of donor cells that contains haematopoietic stem cells (SC). The transplanted inoculum contains mature blood cells of donor origin, including T cells, natural killer (NK) cells and dendritic cells (DC). After transplantation, the progeny of the stem cells repopulate the haematopoietic system of the patient.

46.2.2
Role of Host Dendritic Cells

For a period of time after the preparative treatment, rapid establishment of DC chimerism occurs in hematopoietic cell transplant recipients [54]. Both residual recipient-derived DC that survived conditioning procedures and donor APC derived from the hematopoietic cell graft coexist. The respective role of DC from both origins must be clarified in order to propose new therapies for GVHD (Fig. 46.3).

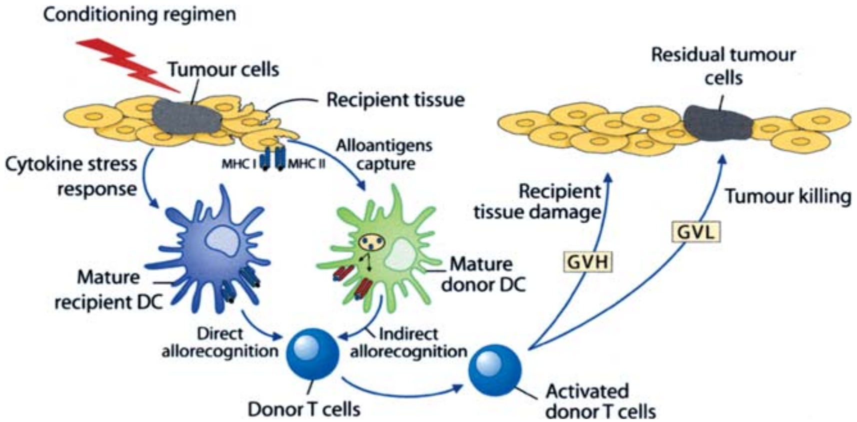

Fig. 46.3 Graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) reaction. GVHD disease is caused by mature T cells in the hematopoietic cell graft attacking tissues of the recipient that have been stressed by the myeloablative conditioning regimen. On the one hand, the cytokine stress response may lead to recipient DC maturation that would directly activate alloreactive donor T cells. On the other hand, the conditioning regimen causing tissue damages would favor the indirect presentation of recipient alloantigens by donor DC to donor T cells. Activated alloreactive donor T cells can also mediate GVL activity by recognition of alloantigens on recipient tumor cells.

46.2.2.1 Host Dendritic Cells in the Initiation and Effector Phases of Graft-versus-host Disease

Host alloantigens directly presented by resident host DC are likely to exert a dominant effect on the activation of donor T cells, resulting in GVHD. Shlomchik et al. significantly contributed to the understanding of the role of host APC in GVHD. In a multiple minor H antigen-mismatched mouse model in which chimeric mice have resident APCs lacking MHC class I due to their β_2-microglobulin mutation and target tissues expressing MHC class I, they demonstrated that host APC play a critical role in the initiation of $CD8^+$ T-cell-induced GVHD [55].

The role of host APC in the effector phase of T-cell-mediated acute GVHD was further documented in subsequent studies. Teshima et al. reported that $CD8^+$ T-cell-mediated acute GVHD was dependent on host APC whereas alloantigen ex-

pression on host target epithelium was not essential for alloreactive T-cell-mediated host tissue damage in the skin, livers, and intestines of recipient animals [56]. Zhang et al. tried to explain why these organs become the main selective targets of activated alloreactive T cells. They discovered that tissue-resident APC control the local recruitment of allo-reactive donor T cells. Using an organ-selective depletion of liver and splenic clodronate-sensitive APC, they observed that the infiltration of $CD8^+$ T cells into the liver during acute GVHD was directly dependent on resident liver APC. In this setting allogeneic $CD8^+$ T-cell trafficking was not affected in the recipient lymph nodes nor in the development of skin lesions [57]. Those data contrasted with those of Everse et al. who previously found in the same model of GVHD that the elimination of residual host APC after the same conditioning treatment of the bone marrow transplant did not decrease the GVHD and even increased mortality [58]. This contradiction was eventually attributed to the presence or absence of $CD4^+$ T cells in the donor T cell preparation. By reconstituting MHC class $II^{-/-}$ recipient mice with either host-derived or donor-derived MHC class $II^{+/+}$ DC or B cells, Teshima's group recently brought new evidence that only host-derived DC are critical for priming donor $CD4^+$ and $CD8^+$ T cells to cause GVHD [59].

46.2.2.2 **Attempts to Eliminate Host Dendritic Cells**

Based on the findings mentioned above, selective targeting of host DC may represent a promising strategy to prevent acute GVHD. The Campath-1G (anti-CD52) monoclonal antibody administration depletes circulating host DC but does not delay donor DC reconstitution [60]. However, CD52 is not expressed on host tissue DC, such as epidermal and intestinal DC [61], which may still activate the GVHD and are also expressed on T cells [62]. Strategy to eliminate host DCs was recently shown to be especially difficult in the case of LC, the major APC in the skin, which is the most frequently affected tissue during GVHD. In contrast with DC population in other organs, LC self-renew locally throughout life without reconstitution from bone marrow precursors unless inflammatory injury occurs [63]. Merad et al. further demonstrated that LC remain host-derived following transplantation unless donor T cells are included in the stem cell inoculum. The replacement of host by donor-derived LC would take place through a two-step process. In the first step, donor T cells directly kill host LC through a CD95-CD95L apoptotic pathway. In the second step, the inflammatory reaction triggered in the skin by donor T cells initiates secretion of chemokines, that presumably attract donor hematopoietic LC-precursors [64].

46.2.2.3 **The Effects of Recipient Conditioning on Dendritic Cell Maturation**

Body irradiation [65] and chemotherapy [66–67] used to condition the recipient of allogeneic cell transplants may induce host DC maturation and thereby promote GVHD. Preparative irradiation has already been shown to induce the release of inflammatory cytokines like TNF-α and IL-1 known to favor DC maturation [68]. In the mouse, recipient's irradiation was also shown to result in significantly in-

creased serum levels of IL-12 and in functional activation of host DC that aggregated in T-cell areas of the spleen within hours [65].

The maturation status of recipient's APC does indeed play a major role in the severity of the GVHD. It was demonstrated that an enhanced allostimulatory activity of host APC in old mice caused significantly worse GVHD than APCs from young recipients, probably through higher TNF-α and IL-12 secretion. This more activated status of APC from old mice is most probably the cause of the more severe $CD4^+$ T-cell-mediated GVHD [69]. Likewise, allogeneic cord blood $CD4^+$ T cells account for a less severe GVHD than adult blood $CD4^+$ T cells, probably by secreting a lower amount of IFN-γ and a higher amount of IL-10. It was demonstrated *in vitro* that this anti-inflammatory cytokine profile of cord blood $CD4^+$ T cells could be reversed when they were stimulated with LPS-treated adult blood DC instead of immature adult blood DC [70].

A new signaling pathway of APC activation during GVHD was recently discovered. Paired immunoglobulin-like receptors, PIR-A and PIR-B, are counteracting receptors for classical and nonclassical MHC class I molecules providing respectively positive and negative regulatory signals to immune cells like B cells, macrophages and DCs. PIR-$B^{-/-}$ mice showed accelerated, lethal GVHD, which was due to augmented activation of recipient DC with concomitant upregulation of PIR-A and increased IFN-γ production by $CD4^+$ and $CD8^+$ T cells. Controlling PIR-A and PIR-B may be therefore essential to minimize GVHD [71].

46.2.2.4 **Reconstitution of Dendritic Cell Content**

The early reconstitution of DC after allogeneic hematopoietic stem cell transplantation was thought to improve survival by stimulating a protective antitumoral response. A clinical investigation recently reported that patients with lower DC counts were found to have significantly worse survival, increased incidence of relapse and unexpectedly a higher incidence of acute GVHD [72].

It was also described that in patients without GVHD, both myeloid DC (DC1) and plasmacytoid DC (pDC, also named DC2) reached consistently high absolute values during the initial phase with a similar time of engraftment [73]. However, it was thought that the reconstitution of pDCs versus myeloid DC content in patient treated with G-CSF might influence the long-term outcome of the engraftment because pDC freshly isolated from human peripheral blood induce antigen-specific anergy in $CD4^+$ T cell lines [74]. The expression of the inhibitory receptors Ig-like transcripts (ILT) 2, 3 and 7 on immature pDCs but not soluble factors like IL-10, TGF-β1 or IFN-α would account for their immunosuppressive properties ([75], and reviewed in [76]. Interestingly, a recent clinical study revealed that the absence of grade II-IV acute GVHD was associated with an improved pDC count. Although pDC count could not predict death from progression or relapse, patients with a "-high" pDC recovery profile had an improved overall survival, in contrast to patients with a "low" pDC recovery profile who had an increased incidence of nonrelapse mortality [77].

46.2.3
Role of Donor Dendritic Cells

The role of donor DC in the indirect presentation of alloantigens during GVHD is unclear. A particular subclass of $CD4^+$ donor DC content in the bone marrow graft before transplantation was reported to be associated with increased leukemia relapse after allogeneic bone marrow transplantation [78]. This decreased GVL effects was associated with a lower incidence of chronic GVHD. Those $CD4^+$ DC present in the bone marrow had the same phenotype as $CD123^+$ type 2 DC progenitors. These particular donor DC2 can be mobilized by G-CSF [79] and their administration in post-transplanted recipients was previously described as responsible for impaired immune recovery [80] and decreased acute GVHD [81]. Likewise, a previously described bone marrow population named $CD8\alpha^+/TCR^-$ facilitating cells was shown to enhance engraftment of hematopoietic stem cells in allogeneic recipient without causing GVHD. This population was recently described as containing plasmacytoid precursor DC that produce IFN-α and TNF-α and that are required to facilitate the engraftment [82].

In the model of $CD8^+$ T-cell-dependent GVHD across minor H antigens, bone marrow transplants from $\beta2m^{-/-}$ donors in which APC are unable to present alloantigens through the indirect pathway, Matte et al. showed that GVHD was less severe. They came to the conclusion that, once initiated by the direct allorecognition by host APC, GVHD is intensified by donor-derived APC. Interestingly, donor APC were not required for $CD8^+$ T-cell-mediated GVL against chronic-phase chronic myelogenous leukemia [83].

It is important to note that the respective role of host- or donor-derived APC may vary according to the type of GVHD (acute or chronic) and of the target organ. For example, it was observed that both donor and host APC can initiate $CD4^+$ T-cell-mediated skin chronic GVHD through a CD80/86-dependent co-stimulatory pathway. However, only donor APC play a dominant role in intestinal chronic GVHD through both CD40 and CD80/CD86 signaling [84].

These data support strategies that target both host and donor APC to prevent GVHD more efficiently.

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47
Dendritic Cells and Transplantation Tolerance

Paul J. Fairchild, Stephen F. Yates and Herman Waldmann

47.1
The Expanding World of Transplantation

Man's dreams of outwitting the natural course of ageing may be traced to classical times. In Greek mythology, Tithonus was granted immortality by Zeus, but not the everlasting youthfulness he craved: instead, afflicted by Zeus' jealousy, Tithonus endured an eternity of the debilitating diseases commonly associated with old age. Although confined to the realms of legend, the moral of the Tithonian dilemma has a strangely contemporary feel, namely, that increased longevity provides no guarantee of greater quality of life.

The past century has witnessed an unprecedented increase in life expectancy in the western world due to improved sanitation and significant medical advances [1]: should the present trend continue, the proportion of the population over 65 years of age will double within the next 20 years while those of 80 years and over will quadruple in number. These sociological changes are likely to be accompanied by a commensurate increase in incidence of disease states such as diabetes, cardiovascular disease and renal failure, threatening to place an unsustainable burden on finite healthcare resources. It is against this background that transplantation medicine has evolved over the past few decades from an experimental procedure with high risks of mortality to the treatment of choice for end stage organ failure. And yet despite this undeniable success, the influence of transplantation remains constrained by a number of factors, not least of which is the availability of donor organs and tissues which has consistently failed to keep pace with the growing demand: of the 6842 patients in the United Kingdom awaiting a suitable donor on 31 December 2001, only 2717 were treated in the following 12 months (according to the UK Transplant Annual Report 2001), illustrating the magnitude of the shortfall.

The restricted availability of viable organs has proven a powerful incentive for investigating novel sources of material for transplantation. While the use of xenogeneic tissues offers the weighty advantages of flexibility and convenience [2], the unresolved issues of hyperacute rejection and potential zoonosis have questioned

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whether the procedure may ever prove acceptable within a clinical context. In contrast, the advent of human embryonic stem (ES) cells has served as a milestone in biomedical science [3–5], offering a potentially limitless supply of therapeutic cell types of human origin. While parallel advances in materials science and tissue engineering may one day permit the routine construction of fully-functional "organoids" [6–8], many chronic and degenerative disease states may already prove amenable to treatment by the replacement of individual cell types, whose premature demise underlies the pathology [9, 10]. Indeed, recent estimates suggest that upwards of 3000 Americans die each day of diseases that may one day be treated through the elixir of regenerative medicine [11].

Although the science of cell replacement therapy (CRT) lags far behind conventional vascularized organ transplantation, recent successes in animal models of human disease offer hope for the eventual treatment of a wide range of disease states. The efficient derivation of substantially pure populations of dopaminergic neurons from pluripotent ES cells has, for example, permitted the symptoms of Parkinson's disease to be treated in both rodents [12] and primates [13, 14]. Importantly, the implantation of this novel source of neurons into the putamen of cynomolgous monkeys, in which Parkinson's disease had been experimentally induced, led to their partial recovery without evidence of the dyskinesias that have so far clouded the use of aborted fetal material in clinical trials. The possibility of effecting myocardial regeneration in the wake of cardiovascular disease has likewise received support from studies in which cardiomyocytes, differentiated from human ES cells, have been shown to integrate functionally into the hearts of recipient pigs where they efficiently assumed the role of a biological pacemaker [15]. Furthermore, the prospect of generating insulin-secreting islet-like structures, responsive to local glucose concentrations [16, 17], suggests novel strategies for the treatment of diabetes, while protocols for the differentiation of mechanoreceptors of the inner ear [18] may help ameliorate chronic hearing loss, the most prevalent decline in function associated with advancing age: even infertility may one day prove amenable to treatment following the controversial derivation of male and female gametes from both mouse and human ES cells [19, 20].

The increasing availability of human ES cell lines [21] has not only expanded the spectrum of diseases for which transplantation may constitute an effective form of medical intervention, but offers prospects for meeting the future demand for cell types by virtue of their capacity for indefinite self-renewal *in vitro*. Nevertheless, CRT, like conventional organ transplantation, is subject to the constraints of histocompatibility which remains the single, greatest barrier to its adoption within the clinic. While for vascularized organs, tissue typing routinely limits disparities at the major histocompatibility complex (MHC), that are recognized as foreign by the recipient, the magnitude of the ES cell bank required to permit any rudimentary matching makes such an approach untenable for CRT [9, 22]. Furthermore, even if matching of MHC genes could be achieved, differences at multiple minor histocompatibility (mH) loci provide ample targets for rejection; given that the identity of selected mH antigens in the mouse has only recently yielded to investigation [23, 24] and that their human equivalents remain largely unknown, treatment re-

gimes currently aim to manage these genetic disparities rather than to seek their elimination. While the widespread use of immunosuppression has helped to achieve this goal over the past 30 years, its long-term use is now known to be detrimental to both recipient and allograft alike. The induction of antigen-specific tolerance to the antigenic disparities therefore offers an alternative approach which avoids the attendant risks of immunosuppression. Here we discuss how the essential role played by dendritic cells (DC) in the initiation of allograft rejection, makes them obvious targets for immune intervention. Furthermore, we review the growing body of evidence that implicates DC in the establishment and maintenance of self tolerance and assess prospects for the strategic exploitation of these unique properties within a transplantation setting.

47.2 The Role of Dendritic Cells in Allograft Rejection

47.2.1 Direct Presentation of Alloantigen

For several decades, DC have been considered the aggressors in transplant rejection by virtue of their unparalleled capacity for the presentation of foreign antigen to naïve T cells [25, 26]. Although conventionally, such antigens are displayed on the surface of DC as peptide fragments bound to products of the MHC [27], early studies of this cell type revealed their unexpected talent for activating T cells from unrelated individuals, by confronting them with whole allogeneic MHC molecules, bound to a diverse repertoire of endogenous peptides. This combination of foreign MHC determinants complexed with peptides to which responding T cells had never been rendered tolerant, proved a potent stimulus for a substantial proportion of the T-cell repertoire, up to 10% of cells becoming activated. Consequently, DC carried over as stowaways in transplanted organs, were considered to represent the principle immunological challenge responsible for precipitating allograft rejection.

The importance of the aptly-named "passenger leukocyte" in this process was elegantly demonstrated by the now classical experiments of Lafferty and colleagues: by maintaining thyroid allografts in culture for extended periods prior to their implantation, their prolonged survival could be readily achieved, even in a fully allogeneic environment. Since the beneficial effects of culture could be reversed by the intravenous administration of peritoneal exudate cells of donor origin, it was assumed that the culture period served primarily to ensure the removal of endogenous DC through their natural turnover [28]. Similar conclusions were drawn from *in vivo* studies in which rat kidney allografts were transplanted into allogeneic recipients under the cover of immunosuppression to prevent their rejection. Having been "parked" in an allogeneic environment for long enough to secure the emigration of resident DC from the parenchyma of the kidney, the allografts were transferred to secondary recipients where they survived long-term without recourse to

immunosuppression [29]. These findings have since been endorsed by the demonstration that DC leaving cardiac allografts migrate to the spleen of recipients where they interact with $CD4^+$ T cells [30]. Furthermore, this productive interaction has been demonstrated by Pietra and colleagues to be wholly sufficient for subsequent rejection. Mice simultaneously deficient in RAG1 and MHC class II genes lack a functional T-cell repertoire, resulting in the unopposed acceptance of cardiac allografts. Critically, the adoptive transfer of $CD4^+$ T cells from syngeneic mice fully restored their ability to reject such grafts: given that MHC class II expression was confined to the donor, direct recognition of alloantigens represented the only possible stimulus for rejection [31].

Although the high precursor frequency of alloreactive T cells results in a potent destructive response that finds no parallel among traditional responses to foreign antigen, early "parking" experiments illustrated the transience of this pathway of sensitization, which was limited by the restricted life span of donor DC. For this reason, it is now accepted that direct presentation of alloantigen fuels the acute phase of rejection, which, if held at bay by immunosuppression, may pose little long-term threat to graft survival. Nevertheless, the demonstration that grafts deficient in donor DC are eventually rejected by the recipient, suggests a further form of rejection reflecting a different underlying physiological process. This premise has since been supported by various studies of the specificity of T cells from patients experiencing delayed rejection of heart, kidney or lung allografts: instead of directly recognizing allogeneic MHC determinants presented by donor DC, these studies showed a progressive increase in the frequency of T cells specific for peptide fragments of these same alloantigens presented in a self-MHC restricted manner, a process now referred to as the indirect pathway of sensitization [26].

47.2.2
Indirect Presentation of Alloantigen

Over the past few years, a growing body of evidence has implicated the recipient's own entourage of DC as accomplices in the rejection process. As a result of their unrivalled capacity for the acquisition of cell debris released from the graft, these cells present such components as though they were conventional foreign antigens, recruiting to the response self-MHC restricted T cells specific for epitopes derived from allogeneic MHC molecules and mH antigens [32]. Donor and recipient DC may, therefore, collude in the process of rejection by mobilizing distinct repertoires of alloreactive T cells.

The importance of the indirect pathway in allograft rejection has been amply demonstrated in a number of compelling studies in the mouse. Valujskikh and colleagues isolated a Th1 cell clone specific for the 58-71 epitope of the H-$2A^k_\beta$ chain when presented in the context of H-$2A^d$. Adoptive transfer of this clone to BALB/c.-SCID mice, lacking a functional T-cell repertoire, rescued their capacity to reject skin grafts of the H-2^k haplotype, recognition of the epitope in a self-restricted fashion leading to infiltration with macrophages whose activation precipitated chronic fibrosis of the graft [33]. Perhaps the most unequivocal demonstration of

the indirect pathway in operation, however, comes from elegant studies by Auchincloss and co-workers who showed that allografts from mice deficient in MHC class II succumbed to the normal course of rejection when transplanted into mice lacking expression of MHC class I. Since the recipients contained only MHC class II-restricted T cells, but the graft itself lacked a source of MHC class II that might be directly recognized, only indirect recognition could ultimately have been responsible for initiating rejection [26, 34]. Whereas these studies illustrate the capacity of $CD4^+$ T cells to operate in isolation to achieve graft rejection, MHC class I-restricted cytotoxic T cells are known to cooperate in this process under normal circumstances. The unique capacity for DC to "crosspresent" endocytosed antigen via MHC class I [35] provides a natural mechanism for inducing their activation and potentiating the response to alloantigen, recent studies having exposed the cellular basis of this novel pathway [36].

The dependence of the indirect pathway of sensitization on the capture of alloantigen by the recipient's own DC, has strong overtones of a conventional immune response to microbial antigens. This contention is supported by the low precursor frequency of cells responsive to epitopes derived from alloantigen, which is more reminiscent of T cells specific for pathogenic epitopes than those directly responsive to unprocessed MHC determinants. Furthermore, the indirect response elicited in patients receiving heart transplants, has recently been shown to be subject to epitope spreading [37], a cardinal feature of deleterious immune responses to defined autoantigens [38], responsible for the sequential recruitment of T cells specific for alternative epitopes and cryptic determinants which amplify the collateral damage [26]. Importantly, the dependence of the indirect pathway on the interaction of donor DC, whose persistence is unchallenged, with a source of alloantigens in the form of a graft, whose persistence is strongly desired, makes this pathway of rejection the more challenging to address since it sets in motion an unyielding response that loses none of its potency with time [34].

47.2.3
The Semi-direct Pathway of Alloantigen Presentation

The discovery of two independent pathways of alloantigen presentation responsible for the sensitization of nonoverlapping subsets of alloreactive T cells, suggests that parallel processes may operate *in vivo* which may have little material influence on one another. Contrary to this expectation, however, $CD4^+$ T cells specific for reprocessed alloantigen presented in the context of self-MHC, were found to provide T-cell help for $CD8^+$ T cells with direct antidonor specificity, amplifying the effector cytotoxic response [39]. Given that these two populations would be expected to recognize their cognate antigen presented by host and donor DC respectively, the question arises as to how they might converge, spatially and temporally, to achieve this outcome.

This paradox has recently been resolved by the description of the so-called semi-direct pathway based on the surprising finding that DC acquire portions of plasma membrane torn from neighboring cells and, with them, integral membrane pro-

Tab. 47.1 Comparison of the attributes of the three pathways of alloantigen presentation that operate following the transplantation of vascularized organs into allogeneic recipients.

	Direct	***Indirect***	***Semi-Direct***
Source of DC	Donor	Recipient	Recipient
Presentation of native alloantigen	Yes	No	Yes
Presentation of processed alloantigen	No	Yes	Yes
Precursor frequency of responding T cells	1:10	$1:10^5$	1:10
Stimulation of self-restricted T cells	No	Yes	Yes
Stimulation of alloreactive T cells	Yes	No	Yes
Duration of the stimulus	Transient	Long-lived	Long-lived
Capacity for crossregulation	No	No	Yes

teins, including MHC determinants (Table 47.1) [40]. Such a light-fingered approach to life appears specific to immature DC, being shared neither by macrophages nor B cells, and offers an explanation for how the same DC may simultaneously present alloantigens by both the direct and indirect routes [41]. That this molecular pick-pocketing by DC may have functional consequences during allograft rejection, has been suggested by Herrera and colleagues who showed that DC cocultured with allogeneic endothelial cells were endowed with the capacity to directly activate an alloreactive T-cell clone *in vitro* [42].

47.2.4
Pathways of Antigen Presentation during Cell Replacement Therapy

Whereas the direct and indirect presentations of alloantigen normally conspire to bring about the rejection of vascularized organs, the use of ES cells as a source of therapeutic cell types offers opportunities for prophylatic intervention in the rejection process, following CRT [9, 10]. While ES cells have been directed in their differentiation along lineage pathways for the generation of numerous cell types of therapeutic value, selection strategies have also been devised to exclude from the resulting progeny DC that would otherwise jeopardize their acceptance by catalyzing the direct pathway of sensitization. By expressing the aminoglycoside phosphotransferase gene under the control of the α cardiac myosin heavy chain promoter, all emerging cell types, with the exception of cardiomyocytes, succumbed to antibiotic toxicity [43]. Using a similar approach, Li and colleagues obtained highly-enriched populations of neurons from differentiating mouse ES cells [44], the use of the insulin promoter having been suggested to provide an equally effective strategy for the selection of pancreatic β cells from such cultures [45].

The control such protocols provide over the composition of the allografted tissues has important implications for CRT, not least of which is the potential for avoiding an acute rejection crisis due to the migration of donor DC to the secondary lymphoid tissues of the host. This suggests that the principle stimulus for rejection under such circumstances would be the indirect presentation of repro-

cessed alloantigens by recipient DC. Although this would be expected to elicit a chronic response, the delay in its onset might provide a valuable window of opportunity for immune intervention that eludes strategies for treating the rejection of vascularized organ allografts. Ironically, while DC have traditionally been considered the aggressors in the process of rejection whose elimination might be expected to have beneficial effects, recent years have witnessed an accumulation of evidence for their complementary role in the induction and maintenance of self-tolerance, suggesting that their administration in a tolerogenic form may actively encourage acceptance of grafted tissues.

47.3 The Role of Dendritic Cells in Self-tolerance

47.3.1 A Cell Type with Two Persona

The undisputed involvement of DC at the very genesis of all immune responses, including transplant rejection, places them in an unrivalled position which might equally be exploited for the induction of tolerance. Just as those operating from within terrorist organizations are best placed to act as informers, so DC may assume conflicting roles according to circumstance, either mounting an all-out offensive or advocating tolerance instead [46]. Early studies exposing the double life of the DC focused on mice transgenically expressing haemagglutinin (HA) in parenchymal tissues including the heart, lungs, salivary glands and male reproductive organs [47]. Curiously, the adoptive transfer of $CD4^+$ T cells, specific for a HA epitope, led to their functional silencing, despite evidence of their transient activation following recognition of HA in a potentially immunogenic form. These findings strongly implicated bone marrow-derived antigen presenting cells (APC) in the induction of peripheral tolerance, a conclusion supported by similar studies in which chicken ovalbumin (OVA) was expressed under the rat insulin promoter to limit its expression to pancreatic β cells and proximal tubular cells of the kidney [48]. That the transfer of OVA-specific $CD8^+$ T cells failed to result in destruction of the target tissues but resulted, instead, in profound peripheral tolerance, defined the cell type responsible as one capable of the crosspresentation of exogenous proteins in an MHC class I-restricted fashion [49], a property now known to be largely confined to DC.

Recent experiments have used more refined experimental models to unequivocally verify the identity of the cell type involved. In an elegant study, Lambolez and colleagues generated mice in which HA was expressed exclusively by B cells and DC: by crossing them onto a RAG-deficient background, expression was further restricted to DC in the thymus and periphery. The adoptive transfer of HA-specific T cells resulted in their initial activation and expansion followed by their precipitous deletion, the few cells that survived having been rendered profoundly nonresponsive to their cognate antigen [50]. Using a wholly different approach,

Finkelmann *et al*. achieved tolerance to rat immunoglobulins of the γ2b isotype by treating mice with the 33D1 monoclonal antibody (mAb), itself a γ2b Ab specific for DC, thereby targeting antigen presentation solely to this cell type [51]. Although these findings are strongly supportive of the tolerogenicity of DC *in vivo*, not all mAbs specific for DC produced the same outcome: far from inducing tolerance, a hamster mAb to CD11c strongly immunized mice to immunoglobulins of hamster origin. These results serve to illustrate the fickle nature of antigen presentation by DC, while raising important questions as to the parameters responsible for tilting the balance in favor of immunity at the expense of self-tolerance. While definitive answers to these questions await consensus, a number of models have been proposed over the last few years that may help resolve this paradox, models which need not be mutually exclusive [46].

47.3.2 Dendritic Cell Subsets Devoted to Tolerance

The suggestion that there may be division of labor between discrete subsets of DC devoted either to tolerance or immunity, is compelling in its simplicity and continues to attract circumstantial evidence from various quarters [52]. Perhaps the most direct demonstration of the dynamic interplay between opposing forces at the very heart of the immune response has been provided by Grohmann and colleagues using an *in vivo* model of tumor peptide presentation. The outcome of antigen recognition appeared to be governed by the balance between distinct subsets of DC under independent control: while IL-12 potentiated the immunogenic subset, as determined by delayed type hypersensitivity (DTH) reactions, interferon-γ (IFN-γ) actively inhibited this effect, augmenting, instead, the tolerogenicity of an alternative subset of DC [53]. Critically, the two populations of cells could be distinguished according to their expression of homodimers of the CD8α molecule, which has subsequently served as a useful phenotypic marker in various studies, likewise purporting to identify cells responsible for the induction of antigen-specific tolerance.

47.3.2.1 Dendritic Cells Expressing CD8α

Although originally thought to be of lymphoid origin [54], it is now widely accepted that $CD8\alpha^+$ DC may develop along either lymphoid or myeloid pathways and that surface expression of CD8α does not, therefore, define their allegiance to a particular ontogenetic lineage [55]. Nevertheless, expression of this co-receptor appears to correlate precisely with the capacity for crosspresentation of exogenous antigen to MHC class I-restricted T cells *in vitro* [56], a role which they also appear to readily perform *in vivo*. Enlightening experiments by den Haan and colleagues demonstrated that administration of splenocytes from $\beta_2m^{-/-}$ mice, which had been loaded with OVA by osmotic shock, led to the efficient priming of OVA-specific CTL in a process dependent on expression, by the recipient, of the transporter associated with antigen processing (TAP), an essential component of the cross-

presentation pathway. Separation of DC from secondary lymphoid tissues, based on their expression of CD8α, revealed how the capacity for crosspresentation segregated wholly with the CD8α^+ subset [57]. Although in this experimental model recognition of cell-associated antigen resulted in priming of MHC class I-restricted CTL, the process of crosspresentation has also been strongly implicated in their tolerization in the periphery [49], providing gratifying symmetry with processes operating centrally within the thymus [58], where the majority of DC express the CD8α molecule [54].

That the mechanism of tolerance induction by CD8α^+ DC is primarily deletional, not only in the thymus but also within the periphery, has been demonstrated in a transgenic mouse model. In experiments aimed at extending early studies in which the expression of foreign antigen was targeted to parenchymal tissues [47, 48], Kurts *et al*. demonstrated the preliminary expansion of antigen-specific CTL upon adoptive transfer to transgenic recipients, followed by their rapid apoptosis in a Fas-dependent manner [59, 60]. Furthermore, in a closely-related study, expression of the herpes simplex virus glycoprotein B (gB) antigen under control of the rat insulin promoter, permitted an MHC class I-restricted gB-specific T-cell hybridoma to be employed as a sensitive read-out for antigen presentation in this model, its activation being monitored by upregulation of the reporter gene, β-galactosidase. Significantly, only CD8α^+ DC, purified from the pancreatic lymph nodes of transgenic mice, were endowed with the capacity to activate the hybridoma, providing compelling evidence that their presentation of antigen *in vivo* was uniquely responsible for establishing peripheral tolerance through activation-induced cell death (AICD) [61].

47.3.2.2 **Plasmacytoid Dendritic Cells**

Plasmacytoid DC are relatively recent players in the turbulent world of DC. Although cells with abundant rough endoplasmic reticulum, reminiscent of antibody-secreting plasma cells, have been recognized within human lymphoid tissues for several decades, their counterparts in the mouse have remained strangely elusive, having only recently been defined as a minor population expressing the B220 isoform of CD45 [62, 63]. Furthermore, their identity as precursors of a previously-unknown DC lineage has only recently come to light [64]: in their immature state, these precursors are set apart from other cell types by their unrivalled capacity for secretion of Type I IFN in response to viral pathogens; indeed, experiments using cells derived from human peripheral blood suggest that they are the principle source of IFN-α released in response to viral challenge, producing between 200- and 1000-fold higher levels of this pro-inflammatory cytokine than any other population of leukocytes [65]. Although the function of the so-called "IFN-producing cells" (IPC) is now well-established, the role these cells adopt within the immune response following their maturation has been rather more difficult to define. Early experiments suggested that IPC may respond to IFN-α in an autocrine fashion, stimulating their maturation into DC capable of presenting viral antigens to naïve T cells, thereby bridging the gap between innate and adaptive immunity [66]. Nev-

ertheless, a growing body of evidence has implicated plasmacytoid DC in covert operations leading to functional tolerance rather than protective immunity [67].

Circumstantial evidence in favor of a tolerogenic role for this DC subset comes from several *in vivo* studies of disease pathogenesis. In a murine model of asthma induced in response to a harmless inhaled antigen, depletion of plasmacytoid DC actively exacerbated the disease, suggesting that, under steady-state conditions, these cells may restrain deleterious immune responses [68], a property which might be exploited by tumors for the evasion of destructive immunity. In support of this prediction, malignant human ovarian epithelial tumor cells have been shown to express high levels of the chemokine stromal derived factor (SDF)-1 known to induce the recruitment and transmigration of plasmacytoid DC precursors to the parenchyma of a developing tumor mass. Here they appear to confer protection from the immune response by stimulating the secretion of anti-inflammatory cytokines, such as IL-10, from invading T cells [69].

These findings serve to illustrate how plasmacytoid DC may employ quite distinct mechanisms for the induction of tolerance, compared to the CD8α^+ subset, which do not rely on the physical deletion of maverick T cells from the peripheral repertoire. Whereas some *in vitro* studies have reported how responding T cells are rendered profoundly anergic following the recognition of antigen presented by plasmacytoid DC [70], others have provided compelling evidence for the polarization of T cells towards a regulatory phenotype, definded by surface expression of CD25, CTLA-4 and the transcription factor, FoxP3. For instance, Martin and colleagues purified plasmacytoid DC from thymus, bone marrow and secondary lymphoid organs by virtue of their expression of B220. Although, following exposure to microbial stimulation, these cells secreted copious IFN-α and acquired a potent immunostimulatory phenotype, under steady-state conditions their encounter with T cells led to profound nonresponsiveness, due primarily to the differentiation of regulatory T cells (Treg) [71]. Paradoxically, others have reported the need for activation of plasmacytoid DC through ligation of CD40 [72] or Toll-like receptor (TLR)-9 [73] before they are able to solicit regulatory activity among responding T cells, the reasons for this discrepancy currently remaining obscure.

The significance of these findings lies in the fact that adaptive Treg induced in the periphery through a variety of experimental regimes [74, 75], have been shown to have a powerful influence on the outcome of organ transplantation in experimental animal models, setting in place a robust form of dominant tolerance [76]. Furthermore, the appearance of cells with the cytokine profile of Treg has been shown to correlate well with long term survival of kidney and liver allografts in human recipients who have fully accepted their grafts despite the withdrawal of immunosuppression [77]. The prospect of harnessing plasmacytoid DC of donor origin to facilitate the deployment of such a network of Treg may, therefore, have important therapeutic implications.

Although, superficially, CD8α^+ DC and DC belonging to the plasmacytoid lineage would appear to employ very different strategies for the induction of tolerance, recent studies have uncovered evidence of a common mechanism. Indoleamine 2,3-dioxygenase (IDO) is responsible for the catabolism of tryptophan and hence

the depletion of this essential metabolite from the tissues in which it is expressed. The immunological credentials of this enzyme were first exposed by Munn and colleagues who demonstrated that its constitutive expression within the placenta protected the developing fetus from rejection by inhibiting the expansion and effector function of alloreactive T cells [78]. More recently, however, the expression pattern of IDO has been shown to correlate with specific DC subsets [79]: while in the human it appears to be confined to plasmacytoid DC [80], in the mouse its expression is less restrictive, being characteristic of both $B220^+$ and $CD8\alpha^+$ DC [81, 82], although the means by which the enzyme becomes active may differ between these cell types. For $CD8\alpha^+$ DC, exposure to IFN-γ alone is capable of stimulating the acquisition of high enzymatic activity [82]; crucially the local depletion of tryptophan helps secure the apoptosis of T cells within the vicinity, contributing to the deletional form of tolerance to which this subset is committed. In contrast, ligation of CD80/CD86 by CTLA-4 stimulates IDO expression by $CD8\alpha^+$ and $B220^+$ DC: since Treg constitutively express the CTLA-4 counter-receptor, they may actively reinforce a state of peripheral tolerance by creating a nonproliferative environment in which T cells, that have evaded apoptosis, may themselves become committed to a regulatory phenotype [83, 84]. It is this regulatory feedback loop that may ultimately underlie the "infectious" nature of dominant tolerance [79].

47.3.2.3 **"Regulatory" Dendritic Cells**

In addition to well-defined subsets of DC represented throughout the periphery, many minor populations of so-called "regulatory" DC have also been described. These cells appear heterogenous with respect to their surface phenotype and function, making their relationships with one another difficult to disentangle. Furthermore, these discrete populations appear largely confined to particular anatomical locations and may, therefore, be actively involved in the amelioration of associated disease states. Various studies have, for instance, investigated the unusual properties of DC resident within the liver. This population may be defined in the mouse by its co-expression of the surface markers Dec-205 (CD205) and B220, a phenotype reminiscent of plasmacytoid DC. Far from secreting IFN-α, however, purified liver DC actively secreted IL-10 upon co-culture with naïve allogeneic T cells, polarizing them towards a Th2 [85] or regulatory phenotype [86]. Significantly, their administration to allogeneic recipients prolonged cardiac allograft survival, strongly implicating this subset of DC in the innate immune privilege enjoyed by hepatic allografts.

The active secretion of IL-10 appears to underlie the regulatory activity of another population of DC defined by its expression of CD4. Studies of experimental autoimmune encephalomyelitis (EAE) in mice have demonstrated how administration of an Fc fusion protein of the autoantigen myelin oligodendrocyte glycoprotein (MOG-Fc) results in its selective uptake by $CD4^+$ DC in the spleens of recipient mice and its presentation in concert with IL-10 secretion, leading to anergy and bystander suppression of encephalitogenic T cells [87]. Crucially, tolerance could not be induced in $Fc\gamma R^{-/-}$ mice but adoptive transfer of $CD4^+$ DC from normal donors

rescued the recipients from the onset of EAE following treatment with MOG-Fc: in contrast, the transfer of conventional myeloid DC or CD8α^+ DC had no discernible effect on disease progression.

Insulin-dependent diabetes has provided an equally rich context in which to study the tolerogenic potential of DC subsets. Naumov and colleagues have reported how CD1d-restricted NKT cells, known to confer protection from diabetes in NOD mice, perform their function partly by recruiting a previously-unidentified population of myeloid DC. When isolated from the draining lymph nodes of the pancreas, these cells were able to abrogate disease onset while DC purified from other lymph nodes had no beneficial effect [88]. In an alternative model of diabetes, in which tissue damage was driven by the recognition of a viral epitope expressed under the control of the rat insulin promoter, protection was conferred on naïve mice by the transfer of a bitypic population of cells co-expressing markers of DC and NK cells [89]. Although the precise mechanisms of tolerance remain to be defined in these models, Tarbell *et al*. have recently demonstrated how DC, differentiated from the bone marrow of NOD mice, can directly expand Treg capable of potently inhibiting diabetes. The resulting Treg exhibited the capacity for linked suppression since they actively blocked responses to multiple autoantigenic epitopes, even though their expansion had been stimulated by a single diabetogenic peptide [90].

Although necessarily anecdotal, these various reports serve to demonstrate how particular populations of DC are especially suited to the induction of tolerance, employing a variety of mechanisms to achieve this goal. Nevertheless, these findings raise important questions as to whether avowedly immunogenic DC may also contribute to tolerance under particular circumstances and, if so, what parameters define the outcome of antigen presentation.

47.3.3
The Maturation Status of Dendritic Cells

47.3.3.1 Immature Dendritic Cells have an Enhanced Capacity to be Tolerogenic

The last few years have witnessed a revolution in our understanding of the physiology of DC and the pivotal role played by maturation in defining their contribution to the immune response. Whereas early studies had largely dismissed immature DC in peripheral tissues, as being wholly preoccupied with antigen acquisition and processing rather than its presentation to T cells, various lines of evidence have converged to give the surprising conclusion that they may be actively involved in the maintenance of peripheral tolerance [91, 92]. This concept owes its origin to studies that extended the pioneering approach of Finkelmann and colleagues [51], in which foreign antigen was delivered to immature DC using, as a convenient vehicle, mAbs specific for Dec-205. By targeting antigen to DC in a noninflammatory context, presentation could be secured without concomitant maturation. By conjugating the Dec-205 mAb to an MHC class II-restricted epitope of hen egg lysozyme (HEL_{46-61}), profound peripheral tolerance was achieved that could not be broken by subsequent immunization with native antigen [93]. Interestingly, the

mechanism of tolerance was found to be primarily deletional, since transient activation of antigen-specific $CD4^+$ T cells was followed by their rapid disappearance from the periphery. Subsequent studies using whole OVA conjugated to the same Dec-205 mAb revealed a similar impact on OVA-specific $CD8^+$ T cells adoptively transferred from T-cell receptor (TCR) transgenic mice. These findings strongly suggested a role for the crosspresentation pathway in the form of tolerance induced, a prediction supported by the requirement for TAP expression by recipients [94]. The involvement of crosspresentation and the ensuing depletion of antigen-specific T cells by AICD, is highly reminiscent of the form of tolerance induced by $CD8\alpha^+$ DC. Accordingly, Dec-205 appears to be preferentially expressed by this subset of DC in secondary lymphoid tissues, suggesting that they are, indeed, the primary recipients of antigen delivery in this system.

While this experimental model may preferentially target $CD8\alpha^+$ DC, the pattern of expression of Dec-205 does not precisely superimpose that of $CD8\alpha$ suggesting that other populations of cells may also collude in the induction of peripheral tolerance. Dec-205 has, for instance, been recognized as an important marker of regulatory DC [95] which has been used successfully to identify tolerogenic DC in the liver [86]. Using DTH reactions as a readout, Mahnke and co-workers showed that administration of OVA coupled to the Dec-205 mAb resulted in robust, nondeletional tolerance accompanied by the emergence of $CD4^+25^+$ Treg [96]. Quite why the administration of the same reagent should lead to two distinct forms of tolerance in different experimental models is currently uncertain, although the distinct nature and anatomical locations of the readouts used may have been significant. Importantly, repetitive stimulation of naïve allogeneic T cells with immature human monocyte-derived DC has likewise been reported to skew commitment towards an IL-10-secreting regulatory phenotype, extending early findings across species barriers and experimental systems [97].

While the clandestine delivery of antigen to DC in a noninflammatory context supports the importance of their maturation status in determining the outcome of antigen presentation, the restricted distribution of Dec-205 among DC would appear to provide as much evidence in favor of a role for discrete subsets in tolerance induction. An entirely distinct experimental system which is not subject to these constraints, has, however, been developed by Probst and colleagues [98]. DIETER mice express three epitopes from lymphocytic choriomeningitis virus (LCMV) which can be inducibly expressed in a DC-specific manner: administration of tamoxifen to transgenic mice resulted in the upregulation of viral epitopes by approximately 5% of immature, resting DC without bias towards any particular subset. Significantly, expression of the epitopes under steady-state conditions resulted in profound tolerance among $CD8^+$ T cells. Although tolerance could not be broken by subsequent infection with LCMV, its induction was inhibited in mice deficient in the gene encoding the negative regulatory molecule PD-1, providing insight into the mechanisms involved [98].

The evident importance of the maturation status of DC on their tolerogenic potential *in vivo*, has been challenged by a conceptual problem: since immature DC are widely supposed to be distributed among interstitial tissues of the periphery,

their opportunities for interacting with naïve antigen-specific T cells would appear distinctly limited. This notion has, however, been challenged by Wilson and colleagues who showed that, while DC migrating to the secondary lymphoid organs were fully mature by standard criteria, those resident within these tissues were phenotypically and functionally immature, placing them in the frontline of the immune system where they might conspire with naïve T cells in the establishment of systemic tolerance [99]. Thus the infrastructure would appear to be in place to make tolerance the default outcome of DC–T cell interactions under steady-state conditions, suggesting that maturation of DC is the essential catalyst that perturbs this *status quo*.

47.3.3.2 **Maturation of Dendritic Cells as a Trigger Point for Immunity**

The importance of DC maturation in the control of destructive immunity has been amply illustrated by the various studies, outlined above, in which antigen delivery was targeted to immature, resting DC *in vivo*, by means of mAbs to Dec-205. While, in each case, profound peripheral tolerance was observed, the co-administration of agonistic mAbs specific for CD40 was sufficient to dramatically alter the outcome, resulting instead in T-cell expansion and acquisition of potent effector function [93, 94]. Likewise, in a TCR transgenic mouse co-expressing viral antigen under control of the rat insulin promoter, administration of the viral epitope resulted in the abortive expansion of antigen-specific T cells which were propelled towards AICD. Co-administration of CD40 mAbs, however, resulted in the rapid onset of diabetes [100]. In mice ubiquitously expressing this same viral epitope, adoptive transfer of antigen-specific $CD8^+$ T cells failed to break the tolerant state, unless accompanied by CD40 ligation, in which case vigorous proliferation of T cells was observed, culminating in the widespread destruction of liver and lymphoid organs [101]. Furthermore, similar findings have been reported in a variety of different settings suggesting the emergence of a universal principle [102, 103]. While this body of evidence offers clear support to the notion that DC maturation acts as a trigger for immunity, it also serves to highlight the particular importance of CD40 signaling in these events: indeed, various studies have specifically identified CD40 ligation as the fulcrum on which the balance between tolerance and immunity appears to pivot [104].

This emerging paradigm in immunology has been strongly supported by studies using, as a source of DC, mice deficient in CD40 expression. Hochweller and Anderton showed how systemic administration of antigen-loaded $CD40^{-/-}$ DC failed to induce T-cell priming and expansion, rendering mice unresponsive to subsequent immunization with the same antigen. When this principle was extended to the use of autoantigens from the CNS, recipients were shown to be protected from the subsequent induction of EAE [105]. Significantly, ground-breaking studies by Martin and colleagues demonstrated how the administration of $CD40^{-/-}$ DC pulsed with keyhole limpet hemocyanin (KLH) could likewise suppress immune responsiveness, even when administered to mice primed 7 days earlier with KLH in complete Freund's adjuvant [106]. These findings were consistent with the abil-

ity of DC to set in motion a dominant form of tolerance through the induction of a regulatory network; accordingly, antigen-specific $CD4^+$ Treg were identified that exerted their suppressive effects through the secretion of IL-10 [106]. Most intriguing of all, however, was the demonstration that, in the absence of CD40 expression, maturation fails to stimulate the acquisition of immunogenicity among DC. This surprising conclusion was drawn from experiments in which maturation was driven *in vivo* by the activation of $V\alpha14^+$ NKT cells in response to the intravenous administration of α-galactosylceramide. Endogenous DC presenting OVA were found to respond by upregulation of MHC determinants and the co-stimulatory molecules CD80 and CD86 but, in the absence of CD40, these cells failed to sustain $CD4^+$ or $CD8^+$ T-cell responses [107].

These findings support the notion that CD40 expression, rather than maturation *per se*, is the critical parameter in defining the outcome of antigen presentation and may help reconcile with the general consensus, studies that implicate so-called "semi-mature" DC in the induction of tolerance [108]. Semi-mature cells have been defined as those expressing a mature phenotype with respect to their expression of MHC class II, but which fail to upregulate co-stimulatory molecules or secrete pro-inflammatory cytokines. While cells with such a profile may correlate *in vivo* with veiled cells of the lymphatics that migrate under steady-state conditions, the differentiation of DC *in vitro* using a cocktail containing granulocyte/macrophage colony stimulating factor (GM-CSF), IL-10, transforming growth factor (TGF)-β and lipopolysaccharide (LPS) appears to arrest DC at a semi-mature stage, endowing them with potent regulatory properties, including the polarization of responding T cells towards a $CD4^+25^+$ regulatory phenotype [109, 110]. While the absence of CD80 and CD86 has traditionally been considered the most important attribute of these cells, recent findings would suggest that the lack of CD40 expression may ultimately prove most relevant to their tolerogenicity.

These findings may help explain the surprising efficacy of co-stimulatory blockade on the survival of organ allografts [111] and are wholly consistent with the observation that mAbs to CD40 ligand (CD40L) induce dominant transplantation tolerance with the distinctive features of regulation, including linked suppression and infectious tolerance [112, 113]. Furthermore, these studies offer hope for the specific use of DC for therapeutic intervention in the process of allograft rejection.

47.4 Exploitation of Dendritic Cells for Transplantation Tolerance

47.4.1 Central Deletion of Alloreactive T Cells

Although the role played by thymic DC in T-cell selection proved highly controversial for many years, various disparate lines of evidence have since converged to ratify their importance in the imposition of self-tolerance on the emerging repertoire through the process of clonal deletion [114]. Expression of the MHC class II deter-

minant, H-2E^d, under the control of the CD11c promoter in C57Bl mice, naturally deficient in H-2E expression, targeted this restriction element to DC of the thymus and periphery. Critically, this restricted distribution was sufficient to purge the repertoire of Vβ5$^+$ and Vβ11$^+$ T cells, known to be responsive to H-2E^d, providing unequivocal evidence for the involvement of DC in this process [115]. Fetal thymus organ cultures (FTOC) have likewise proven an effective way of dissecting the relative contributions of the various stromal elements to positive and negative selection pressures, an approach amply illustrated in our own laboratory using A1 mice. This strain transgenically expresses a TCR specific for an epitope from the male antigen, Dby, in the context of H-2E^k: colonization of thymi excised from female embryos with DC differentiated *in vitro* from male bone marrow precursors, results in widespread deletion of CD4$^+$8$^-$ thymocytes, reducing the proportion of cells acquiring a functional phenotype to levels evident among control male fetal thymi, cultured in parallel (Fig. 47.1).

That the physical elimination of T cells bearing undesirable specificities is an irreversible process, offers an attractive form of tolerance which many have sought to exploit in the service of organ transplantation. Importantly, colonization of the thymic medulla *in vivo* with allogeneic DC of donor origin may be achieved by the administration of donor bone marrow as a way of establishing mixed chimerism

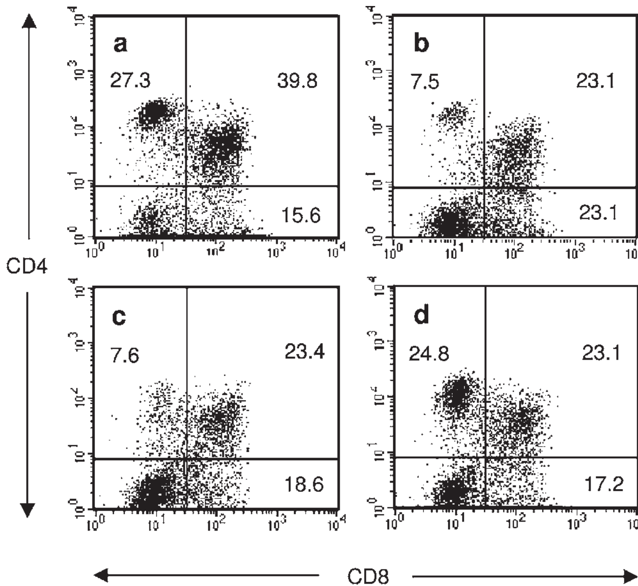

Fig. 47.1 Presentation of endogenous male antigen by bone marrow-derived DC induces clonal deletion of CD4$^+$8$^-$ thymocytes in organ cultures of A1 fetal thymi. (a and b): Distribution of thymocyte subsets in female (a) versus male (b) fetal thymi, demonstrating negative selection to occur at the transition to a mature phenotype. (c and d): Clonal deletion in female thymi induced by colonization with male (c) but not female (d) DC differentiated from the bone marrow of syngeneic mice.

[116]. This strategy has already proven capable of eliminating alloreactive T cells and securing long-term acceptance of MHC-mismatched skin grafts, known to represent a potently-immunogenic challenge. Furthermore, these experimental protocols have been successfully transferred from rodents to nonhuman primates, suggesting possible clinical relevance [117].

While donor bone marrow may legitimately contribute to all hematopoietic lineages within the recipient, several lines of evidence implicate DC, resident within the thymus, as responsible for the subsequent state of tolerance. In pioneering studies, Gandy and co-workers progressively fractionated the donor inoculum in order to identify the component responsible for facilitating bone marrow engraftment. This approach revealed an absolute requirement for cells that lacked TCR yet co-expressed CD11c and CD8α, a phenotype highly suggestive of DC actively involved in deletional tolerance. Furthermore, the site of tolerance induction was demonstrated to be the thymus in experiments involving the direct intrathymic injection of peptides derived from alloantigens, an operation that mimicked the tolerance induced through mixed chimerism [118]. Since the same outcome could be obtained by the intrathymic delivery of purified thymic DC [119], the cellular basis of the tolerant state appears to be wholly dependent on DC and the contribution they make to repertoire selection centrally within the thymus.

Although mixed chimerism shows potential for the treatment of allograft rejection, it is, nevertheless, subject to various constraints. Arguably the greatest drawback has been the conditioning regime required to permit the acceptance of the donor bone marrow, which has traditionally required extensive myelo-ablation. Some of the related toxicity has recently been circumvented by the advent of protocols employing so-called “megadoses” of hematopoietic stem cells in conjunction with co-stimulatory blockade, so as to blindfold endogenous DC to the presence of the bone marrow graft. Even when using doses of bone marrow some 13-fold higher than previously described, however, tolerance may only be achieved in 50% of cases, a success rate too low for its wholesale adoption within the clinic. Secondly, by being dependent on thymic function for its success, the approach is necessarily untenable following thymic involution, placing important age-related restrictions on those that might benefit; given that chronic and degenerative disease states are significantly more prevalent in the elderly, this constraint may make mixed chimerism the preserve of those experiencing the early onset of symptoms. Even when performed successfully in patients whose thymus remains functional, the form of tolerance is inherently recessive, requiring the ongoing elimination of alloreactive T cells from the pool of recent thymic emigrants. Consequently, the unscheduled demise of donor DC from the thymus of the recipient would herald the loss of the tolerant state. In contrast, regulation is a dominant form of tolerance which, by virtue of the “infectious” manner in which it operates, is unperturbed by the continual emergence of alloreactive T cells from the thymus [74]; it is, therefore, this form of immune intervention that currently offers the greatest potential for the induction of transplantation tolerance.

47.4.2
Peripheral Regulation of Alloreactive T Cells

The demonstration that immature DC may polarize the T cells they engage towards a regulatory phenotype has opened the way for their exploitation in a therapeutic context, the potential of this approach having recently found support in the form of early human trials. Dhodapkar and colleagues administered immature monocyte-derived DC, pulsed with either KLH or influenza matrix peptide (MP), to two healthy volunteers. Interestingly, rather than priming individuals to these antigens, this experimental regime led to the emergence of regulatory T cells. Although these cells were $CD8^+$ rather than conventional $CD4^+25^+$ Treg, they were, nevertheless, capable of suppressing MP-specific T cells, previously isolated from the peripheral blood, in a process dependent on their secretion of IL-10 [120, 121]. The relevance of such an approach to the establishment of tolerance across MHC barriers as a prelude to transplantation, has been reported in various studies in mice, epitomized by the experiments of Lutz and co-workers. This group showed how the adoptive transfer of phenotypically-immature bone marrow-derived DC resulted in the prolongation of cardiac allograft survival from 8 to more than 100 - days when the conditioning regimen was applied 7 days prior to transplantation [122]. While such studies have provided an important proof of principle, the maturation state of DC is known to be highly sensitive to a plethora of stimuli raising concerns that the state of tolerance provoked by immature DC may be inherently fragile and may even be reversed, should they subsequently mature following administration *in vivo*. Accordingly, significant effort has been invested over recent years into protocols that might arrest DC at an immature stage of their development, conducive to tolerance induction.

A variety of pharmacological agents have been employed in this context as a result of their capacity to interfere with the process of maturation (for a more detailed account please see the related Chapter 30). There is, for instance, a long history, dating back more than a decade, documenting the effects of IL-10 on the physiology of DC. Although this noninflammatory cytokine was originally shown to inhibit maturation [123, 124], recent data suggest the "alternative activation" of DC in a manner similar to that reported for macrophages, rendering them inherently tolerogenic. In support of this contention, Wakkach *et al*. have shown how murine DC differentiated from bone marrow precursors in the presence of IL-10 are characterized by a distinctive $CD11c^{lo}$ $CD45RB^{hi}$ phenotype and are capable of polarizing responding T cells towards a regulatory phenotype of the Tr1 subset, whose secretion of IL-10 sets in motion a powerful form of dominant tolerance [125].

Perhaps the most promising pharmacological agent to have emerged over recent years is, however, 1α,25-dihydroxyvitamin D_3 (VD_3), the physiologically-active form of vitamin D_3. Direct administration of this agent to recipients in conjunction with mycophenolate mofetil (MMF), was found to be accompanied by the emergence of $CD4^+25^+$ Treg and subsequent acceptance of fully mismatched mouse islets. Importantly, the tolerant state resisted the infusion of naïve splenocytes and paved the way for the subsequent acceptance of cardiac allografts from the same

donor strain, compelling evidence consistent with its dominant nature [126]. Nevertheless, since VD_3 has been shown to have a direct impact on T cells, even in the absence of APC [127], this study failed to identify the DC as the primary target of pharmacological intervention. Related studies by Penna and colleagues, using human monocyte-derived DC, have, however, resolved this issue by showing the phenotypic arrest of DC at an immature stage in response to VD_3 and their protection from LPS-driven maturation [128]. Furthermore, when administered *in vivo*, murine DC, conditioned *ex vivo* with VD_3, significantly prolonged skin allograft survival, strongly endorsing the use of such an approach in a therapeutic setting [129].

While the use of DC for the induction of transplantation tolerance has traditionally focussed either on deletion of alloreactive cells or their active restraint by the imposition of a regulatory network, pioneering studies by Strom and colleagues have revealed how these two processes may work in concert to achieve a profound state of transplantation tolerance [130]. In situations in which AICD of alloreactive T cells was inhibited, tolerance across MHC barriers could not be achieved, even when Treg had been induced [131]. Conversely, experimental programmes that enhanced the apoptosis of responding T cells actively promoted dominant tolerance [132], strongly suggesting that a reduction in the burden of alloreactivity in the periphery of recipients, helped tilt the balance in favor of regulation [133]. Given that these two forms of tolerance may conspire to achieve the long-term acceptance of organ allografts, future protocols using DC as a conditioning regimen might be wise to reflect this finding. It may, for instance, prove beneficial to simultaneously enlist the properties of $CD8\alpha^+$ DC for the purpose of deletion and immature DC in order to polarize remaining T cells towards a regulatory phenotype.

47.4.3
Reinforcing a Tolerogenic Phenotype by Genetic Modification

While pharmacological intervention holds promise for the generation of DC indefinitely suspended at an immature stage of their life cycle, pharmacological agents necessarily exert multiple effects on gene function, not all of which are beneficial: it is possible, for instance, that exposure to nonphysiological concentrations of compounds may lead to the accumulation of mutations and an increased likelihood of transformation. An alternative approach, adopted by many groups, has, therefore, been the genetic modification of DC in order to specifically confer on them desirable properties. Although emerging new technologies, such as the serial analysis of gene expression (SAGE), have begun to highlight specific genes associated with pharmacologically-treated DC [134], a comprehensive gene signature remains to be defined. Efforts to date have, therefore, concentrated on the expression of individual genes rationally selected to facilitate tolerance induction.

The forced expression of a viral homolog of the mammalian IL-10 gene has, for instance, succeeded in creating a milieu, in the vicinity of administered DC, conducive to transplantation tolerance [135]. Perhaps the best-documented approach, has, however, been the constitutive expression of FasL creating so-called “killer” DC, able to induce apoptosis among alloreactive T cells. This strategy owes its ori-

gins to the finding that AICD by CD8α^+ DC is dependent, at least in part, on the Fas-FasL pathway [60] and that the engraftment of allogeneic bone marrow shows a similar requirement for FasL expression by the donor inoculum [136]. Accordingly, Matsue and colleagues have shown that DC transduced with FasL were competent to delete T cells *in vivo* and establish a state of antigen-specific nonresponsiveness if administered to recipients prior to sensitization [137]. Likewise the use of "killer" DC by Zhang *et al.* generated tolerance across both mH and MHC barriers [138], findings which have since been extended to show the prolonged survival of fully MHC-mismatched cardiac allografts [139].

Despite these successes, many problems need to be resolved before genetic modification may be routinely adopted in a therapeutic context. Currently, the transfection efficiencies for DC using electroporation or lipofection are extremely low, necessitating the use of adenoviral or lentiviral vectors for the introduction of heterologous genes. Nevertheless, most, if not all, viral vectors have a deleterious impact on DC which, as sentinels of the immune system, have evolved to sense the presence of viral subterfuge. Consequently, many vectors may corrupt the very functions of DC that make them unique, most commonly inducing their premature maturation [140], an outcome likely to be counterproductive if tolerance is desired. Furthermore, the reproducibility between batches of genetically-modified DC is low, making quality control a significant issue, should translation to the clinic ever be pursued.

47.5 Prospects for the Induction of Tolerance via the Indirect Pathway

Whether subtly modified by genetic means or conditioned by pharmacological intervention, prospects for harnessing the properties of DC to induce tolerance are encouraging, at least among T cells directly recognizing the alloantigens they express. In contrast, the establishment of tolerance among T cells responsive to the same alloantigens presented indirectly as peptide fragments in a self-MHC restricted manner poses special problems that are conceptually difficult to address: no matter how tolerogenic DC may be, they have no capacity to influence the tolerogenicity of recipient DC, responsible for their uptake. The issues involved have been amply illustrated by experiments employing DC constitutively expressing FasL. Whereas their administration was found to promote the depletion of alloreactive cells through the direct route, "killer" DC proved wholly incapable of preventing allograft rejection, most likely as a result of their lack of impact on the self-restricted T-cell repertoire [137]: even in experiments in which allograft survival was prolonged, chronic rejection was unimpaired, strongly suggesting that the indirect pathway of alloantigen presentation remained firmly intact [139]. In the light of such results, it is perhaps pertinent to ask whether the administration of exogenous DC can ever exert sufficient influence over both the direct and indirect presentation of alloantigen to restrain both acute and chronic rejection.

Ironically, it is the semi-direct pathway of presentation that offers the greatest hope for crossregulation between T cells of direct and indirect specificity (Table 47.1). Donor DC, prevented from maturing to render them tolerogenic, may, for instance, induce Treg with direct specificity for the alloantigens they express. Rather than waning with time following the demise of donor-derived passenger leukocytes, their activity might be sustained by recipient DC that have acquired whole alloantigen from endothelial cells of the graft: since these DC will inevitably present the same alloantigens via the indirect pathway, they may inadvertently form a rendezvous for Treg of direct specificity and naïve T cells indirectly recognizing alloantigen [41]. Under such circumstances, potentially-aggressive T cells may themselves adopt a regulatory phenotype in accordance with experiments showing that linked suppression can operate through the indirect pathway [141]. That, in principle, such a scheme may prove effective has been supported by the work of Mirenda and colleagues who were able to establish dominant tolerance in LEW rats to cardiac allografts from AUG donors by the simple expedient of administering stably immature DC from (LEW x AUG)F_1 rats, guaranteed to present alloantigens simultaneously via direct and indirect means, thus mimicking the semi-direct pathway (Fig. 47.2) [142].

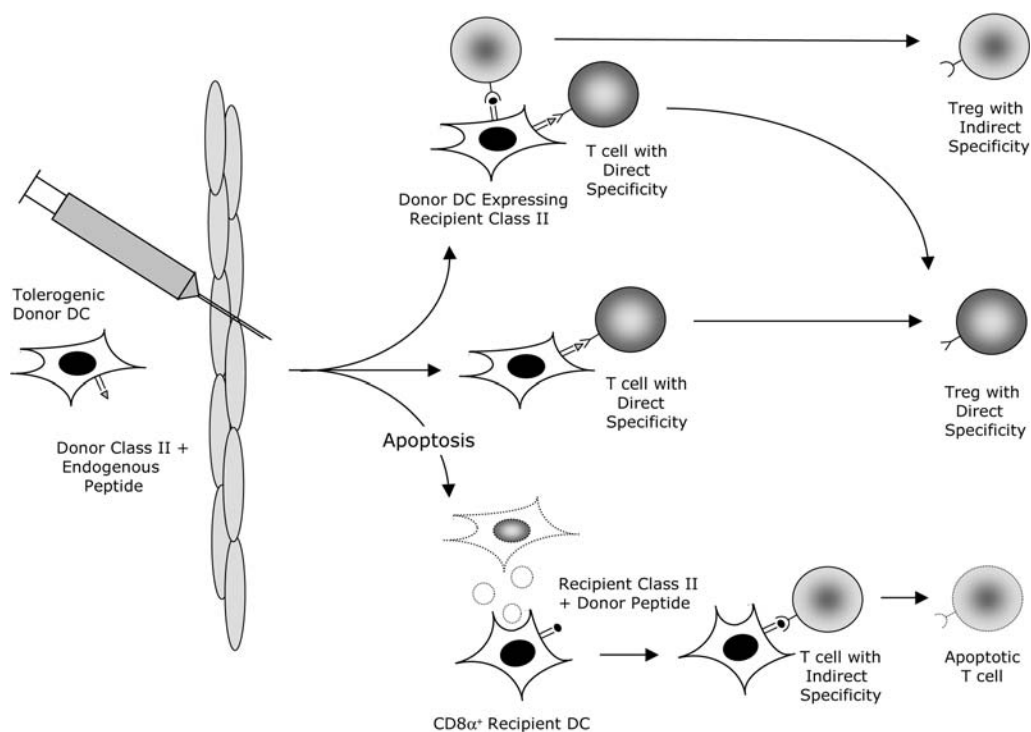

Fig. 47.2 Scheme depicting the possible ways in which administration of tolerogenic DC of donor origin might induce transplantation tolerance among T cells displaying either direct or indirect specificity for alloantigen.

Another source of hope that the indirect pathway may be amenable to immune intervention by DC, has emerged from recent findings of the anti-inflammatory properties of apoptotic cells, both directly, through their release of IL-10 and indirectly via their stimulation of TGF-β secretion from the macrophages that ingest them [143]. Importantly, endogenous, resting DC are particularly adept at the uptake of apoptotic cells by CD36 and $\alpha_V\beta_5$ integrins, which are downregulated upon maturation [144]. Unlike the encounter with necrotic cells, however, apoptotic bodies do not appear to induce DC maturation, allowing their components to be presented in a tolerogenic fashion. That the uptake and presentation of apoptotic cells represents a physiological pathway for the maintenance of peripheral self-tolerance has been strongly suggested by the landmark experiments of Huang and colleagues who traced the transport of self proteins expressed by epithelial cells of the gut to the mesenteric lymph node in the form of apoptotic material carried by immature DC under steady-state conditions [145]. Similar findings have been reported in the skin where Langerhans cells, on leaving the epidermis en route to the draining lymph nodes, have been shown to contain melanin granules acquired from neighboring cells following their apoptosis [146].

Although the constitutive uptake of apoptotic material by resting DC has been proposed to contribute to self tolerance [92, 147], direct evidence has only recently come to light [148, 149]. Ferguson and colleagues showed how the intravenous administration of syngeneic splenocytes, coupled with foreign antigen, led to antigen-specific tolerance, the potency of which could be enhanced up to 100-fold by rendering the splenocytes apoptotic [149]. In this and other studies, $CD8\alpha^+$ DC were found to be responsible for the uptake and presentation of dying cells, suggesting that a deletional form of tolerance might be involved [150]. The infusion of immature donor-derived DC may, therefore, offer the prospect of inducing regulation through the direct pathway by polarizing alloreactive T cells towards Treg, while simultaneously fuelling a deletional form of tolerance among T cells of indirect specificity by providing apoptotic material for presentation by resident $CD8\alpha^+$ DC. In this respect, the administration of DC as a conditioning regimen prior to organ transplantation may mimic the beneficial effects of a donor-specific blood transfusion, which has likewise been shown to induce tolerance primarily through the indirect pathway [151].

The need to induce tolerance among T cells specific for alloantigen presented indirectly, is not only critical to counteract the chronic rejection of vascularized organs, but assumes even greater importance in the emerging field of CRT. In this case, the level of control that may be exerted over the differentiation of ES cells is likely to ensure the exclusion of DC from their progeny that might stimulate the direct pathway of allorecognition. Consequently, it is the indirect route that is likely to play the most significant role in rejection of replacement tissues, raising questions as to how this might be effectively controlled.

47.6 Immune Intervention in Cell Replacement Therapy

The unique origin of cell types differentiated *in vitro* from ES cells offers unparalleled opportunities for intervention to mitigate the impact of immune recognition by the recipients of CRT. In particular, the pluripotency of ES cells, which makes them an ideal source of replacement tissues, might also offer a solution to the issue of rejection by permitting the differentiation of the very cell types involved in self tolerance, which, by default, will express all transplantation antigens to which tolerance must be established [9, 10]. Furthermore, the capacity to separate, temporally, these two differentiation programmes offers the prospect of preemptive tolerance induction in advance of CRT, an advantage that is denied the recipients of vascularized organs from cadaveric donors. The unique opportunity this therapeutic window provides might be exploited in one of two ways.

47.6.1 Generation of Hematopoietic Stem Cells (HSC) for Mixed Chimerism

Mouse ES cells have traditionally provided an experimental system amenable to the study of hematopoiesis, since commitment to the mesodermal germ layer represents a common pathway during their chaotic differentiation *in vitro* [152]. More recently, studies of ES cells of human origin have likewise reported their propensity to support hematopoiesis [153], raising the prospect of their exploitation as a ready source of HSC for the establishment of mixed chimerism. While undoubtedly an attractive possibility, a number of issues currently limit such a strategy.

It has long be appreciated that HSC from either fetal or adult sources exhibit the capacity for the long-term repopulation of irradiated recipients but that HSC derived from the visceral yolk sac of early embryos lack this propensity. Critically, this limitation is shared by HSC differentiated from ES cells, most likely as a result of their reticence to seek out and colonize appropriate niches, once administered *in vivo*. Müller *et al.* demonstrated how HSC obtained from the *in vitro* differentiation of mouse ES cells supported only limited hematopoiesis following their introduction into normal recipients [154]. Crucially, these cells contributed solely to the lymphoid lineages, failing to generate either the DC required to establish a state of mixed chimerism or any other cells of myeloid origin. While problems associated with the migration of ES cell-derived HSC have been partially circumvented by their direct administration to the bone marrow of recipients [155], such a regime is unlikely to prove clinically acceptable. Furthermore, difficulties inherent in the expansion and maintenance of HSC *in vitro* make it unlikely that sufficient numbers of cells could ever be obtained to deliver the megadoses required to achieve mixed chimerism in the absence of myelo-ablation. In an attempt to overcome many of these issues, our own laboratory has focussed on elucidating the differentiation pathway of DC from ES cells with a view to their use as a tolerizing regime in advance of CRT [9].

47.6.2
Generation of Dendritic Cells for Tolerance Induction

Embryoid bodies (EB) are macroscopic structures formed by the proliferation and concomitant differentiation of ES cells maintained in suspension culture and mimic, albeit imperfectly, development of the early embryo in all but pattern formation of the resulting tissues, which are inherently chaotic in their organization. Importantly, EB represent a microenvironment conducive to hematopoiesis since they form cyst-like structures, similar in their composition to the visceral yolk sac: by maintaining them under carefully controlled conditions *in vitro*, in the presence of growth factors implicated in DC ontogeny, we have demonstrated the feasibility of deriving almost limitless numbers of primary, untransformed DC from mouse ES cells [156, 157]. Cultures of so-called esDC are highly reminiscent of DC differentiated *in vitro* from bone marrow progenitors (bmDC) (Fig. 47.3a), but display additional properties which make them ideal candidates for therapeutic purposes [158].

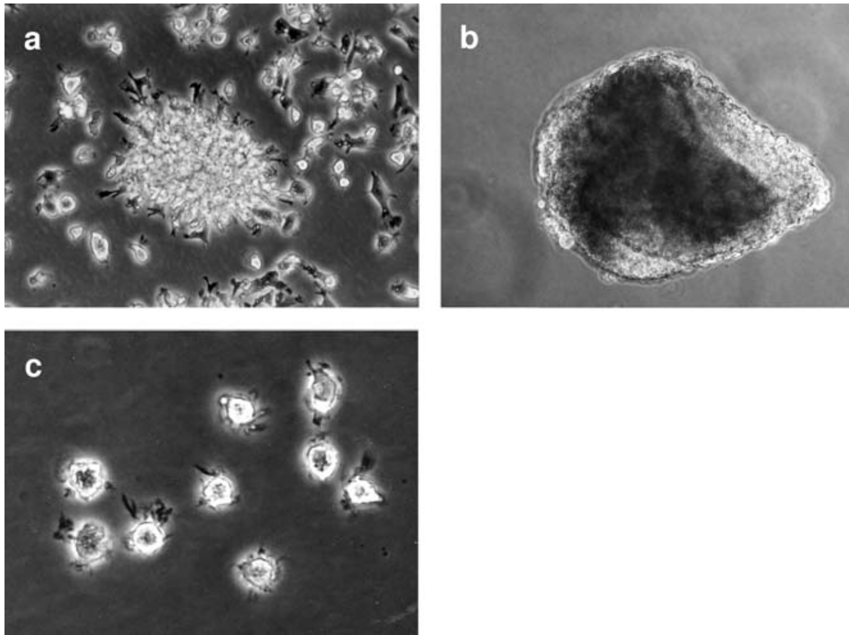

Fig. 47.3 Photomicrographs of esDC differentiated from mouse (a) and human ES cells (b and c). (a): Immature murine esDC forming clusters of cells highly reminiscent of DC generated from mouse bone marrow progenitors (Magnification ×20). (b): Embryoid body derived from human ES cells maintained in suspension culture for 6 days prior to the differentiation of DC (Magnification ×10). (c): Human esDC showing the veils of cytoplasm and dendrites characteristic of this cell type (Magnification ×40).

Firstly, esDC are characterized by a capacity for expansion far greater than described for their bone marrow-derived counterparts: an individual EB, derived from the differentiation of a single ES cell, may generate upwards of 3×10^7 esDC over a three-week period which, once established as a primary culture, may be harvested repeatedly before eventually succumbing to quiescence. Furthermore, esDC differ from conventional sources of DC with respect to their maturation status: while they respond to exogenously-added stimuli, such as LPS, by assuming a mature phenotype and migrating to secondary lymphoid tissues *in vivo*, in the absence of such a challenge they remain uniformly immature, lacking any propensity for the spontaneous maturation that accompanies the derivation of bmDC and questions the expediency of their use for the induction of peripheral tolerance [157]. Perhaps the most gratifying feature of these cells, however, is the opportunity they offer for the rational design of DC endowed with specific properties through genetic modification.

Transgenes or silencing constructs introduced into the parent ES cell line, have been shown to be faithfully expressed by esDC downstream of the differentiation pathway, providing significant advantages over current strategies aimed at the genetic modification of DC. Since ES cells are rather more amenable to transfection than terminally-differentiated DC, approaches such as lipofection may prove wholly adequate, circumventing the need for viral vectors that might subsequently corrupt DC function. Furthermore, the propensity for cloning of ES cells at the single cell level, has been harnessed for the generation of lines of esDC uniformly expressing a desired mutant phenotype, thereby greatly surpassing current transfection efficiencies [158, 159]. The option to genetically manipulate esDC is an attractive one since it may facilitate the expression of genes associated with pharmacologically-treated DC in order to reinforce their tolerogenicity. The introduction of a single MHC class II gene of recipient origin, may also be essential to confer on the resulting cells the capacity to present alloantigen by both direct and indirect pathways (Fig. 47.2).

Perhaps the most appealing feature of esDC, however, is the permanent resource that a clonal population of genetically-modified ES cells provides, from which “designer” DC may be differentiated on demand. Importantly, the level of reproducibility of the phenotype and function of DC differentiated from this source has been shown to offer unparalleled consistency between batches [10]. In a clinical setting, this may permit essential quality controls to be conducted with respect to the functional capacity of the DC to be administered, the likelihood of their transformation *in vitro* and the potential they hold for harbouring human pathogens. Together with preliminary findings from our laboratory that support the feasibility of adapting protocols devised in the mouse for the differentiation of esDC of human origin (Fig. 47.3b–c), these advantages may make CRT the future platform of choice from which to launch the clinical use of DC in the establishment of transplantation tolerance.

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48
Dendritic Cells, Immune Regulation and Transplant Tolerance

Giorgio Raimondi and Angus W. Thomson

48.1
Introduction

Since the discovery that allograft rejection is due to activation of the recipient's immune system, evidence has accumulated of a complex immune response that entails extended dynamic interactions between its cellular and humoral components. These findings underscore a central role of dendritic cells (DC) in orchestrating and controlling alloimmune reactivity. Considerable evidence indicates that DC are the principal instigators of rejection, but accumulating data also depict DC as critical regulators of alloimmune responses. This knowledge has driven efforts to identify strategies that exploit the tolerogenic properties of DC to promote transplant tolerance. Herein we review evidence that DC can function as regulators of immune reactivity in relation to organ transplant outcome. Special emphasis will be placed on the significance of the "tolerogenic" functions of DC that provide the basis of current experimental DC-based therapies for promotion of transplant tolerance.

48.2
Dendritic Cells and Initiation of the Rejection Response

The generally accepted paradigm of activation of an adaptive immune response requires presentation of foreign antigens (Ag) by "professional" Ag-presenting cells (APC). DC are a heterogeneous leukocyte population (derived from $CD34^{+}$ stem cells) with morphology (veil-like processes and dendrites) and mobility that are well-suited to Ag capture, processing, and presentation. DC present Ag to the rare T cells that express specific receptors that recognize Ag-derived peptides bound to major histocompatability complex (MHC) molecules. DC show several distinctive features that justify their classification as "professional" APC. These include: (i) ability to stimulate naïve $CD4^{+}$ and $CD8^{+}$ T cells efficiently, (ii) capacity to transport Ag from peripheral tissues to T-cell areas of secondary lymphoid organs (where

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naïve T cells recirculate), and (iii) ability to "cross-present" foreign Ags in the context of MHC class I molecules to Ag-specific $CD8^+$ T cells [1]. More specific details concerning their properties/abilities as APC can be found in other chapters of this book.

The relation between these characteristics of DC and the initiation of allograft rejection is determined by two factors: (a) the distribution and (b) the activity of the DC. In the normal steady state (absence of inflammation or "danger"), DC reside in the interstitial spaces of most peripheral tissues, including the commonly-transplanted organ/tissues, with the exception of the central cornea and brain parenchyma. DC are also abundant in T-cell areas of the spleen and lymph nodes, and in the medulla of the thymus. The significance of this distribution has been clarified with the elaboration of a general paradigm for DC activity (based on the functions they exhibit following their isolation and reinfusion into experimental animals): DC acquire Ags in the periphery by phagocytosis and macropinocytosis, and migrate to T-cell areas (in spleen via the blood; in lymph nodes via the lymph), where they can either initiate an immune response against the Ags they present, or simply die [2]. Death is followed by re-presentation of proteins from the dying DC by resident DC in the lymphoid organ. This phenomenon plays an important role in the regulation of immune activation and tolerance induction. In transplant models, DC derived from allogeneic tissues and bearing donor MHC antigens, can be identified in peripheral lymphoid organs, where they can stimulate T cells directly.

A second aspect of the general paradigm for activation of an immune response is the requirement for "mature" APC that present foreign Ag to initiate the adaptive response. During their lifetime (characterized by considerable turnover), DC pass through various phenotypic stages, generally referred to as "maturation states". DC in normal blood and nonlymphoid tissue are regarded as "immature". This state is characterized by the ability of DC to internalize exogenous Ag (through phago- or macropinocytosis and various receptor-mediated endocytic processes) and to process and load the Ag-derived peptides onto intracellular MHC molecules. This is associated with only weak ability to stimulate T-cell activation/proliferation, due to low cell surface expression of MHC and co-stimulatory/accessory molecules (e.g. CD40, CD80, CD86). These immature DC are equipped with various cell surface receptors, ligation of which initiates a signaling pathway, characterized by nuclear translocation of the gene transcription regulatory factor, nuclear factor (NF)-κB, that initiates DC maturation. These cell surface receptors interact specifically with exogenous and endogenous mediators released into the microenvironment during inflammation: (i) bacterial or viral components (e.g. LPS, CpG, or double-stranded RNA), recognized by Toll-like receptors (TLR) 2, 3, 4, 7 and 9; (ii) pro-inflammatory cytokines (e.g. GM-CSF, IL-1β, TNF-α, IFN-α) and cyclooxygenase metabolites (e.g. prostaglandin E2), recognized by their specific counter-receptors; (iii) specific ligands, expressed on the surface of "activated" cells (T cells, platelets and mast cells), that are recognized by molecules of the tumor necrosis factor receptor (TNFR) family on the DC surface (e.g. CD40, TNFR, receptor activator of NFκB [RANK]). As a result of these interactions, DC are stimulated to become "mature" APC. They briefly increase their endocytic processes (in order to

properly sample the "suspected" area) [3], then significantly downregulate these activities, translocate peptide loaded-MHC molecules to the plasma membrane and upregulate cell surface T-cell co-stimulatory molecules (CD80, CD86, OX40 ligand [L] and inducible co-stimulator [ICOS] L) and intercellular adhesion molecules (CD54 and CD58), necessary for assembly of the immunological synapse [1]. Furthermore, as they mature, DC produce pro-inflammatory cytokines and increase surface expression of the CC chemokine receptor CCR7, which enables their traffic to T-cell areas of secondary lymphoid organs, in response to the CCR7 ligands CCL21 and CCL19. Therein, DC encounter rare, Ag-specific T cells, and function as powerful naïve and memory T-cell-priming APC.

The act of transplantation by itself triggers the maturation and migration of graft-resident and graft-infiltrating DC [4] where the eliciting stimuli, according to the "danger theory" model [5], are probably innate "danger signals" (such as heat shock proteins [HSP], uric acid, and high mobility group box 1 molecule [HMGB1]) released in response to ischemia-reperfusion injury and surgical trauma. The exact stimuli responsible for DC maturation after organ transplantation have not been fully elucidated, but studies of rejection using combinations of donor and recipient Myd88 knockout animals (Myd88 is a key signaling factor for many TLR) have clearly indicated that multiple (dominant and complementary) pathways are involved in the initiation of the response. In this regard, two recent studies have delineated the distinct aspects of initiation of the rejection response [6, 7]: both studies involved transplants using T-cell deficient mice as recipients, which allowed the acceptance and healing of the grafts. In both cases, the injection of T cells (or fetal liver stem cells) after a period sufficient for the graft to heal, thus in the ostensible absence of danger, drove prompt rejection of the transplanted tissue. Two explanations have been offered for this unexpected result. The first is related to the observation of a differential level of expression of some housekeeping genes in the healed graft. This could indicate that, even if not observed at the phenotypic level, a state of inflammation may be maintained in the tissue and that interaction with transferred T cells may allow the conventional process of their activation to take place. The second interpretation derives from a still controversial exception to the classical paradigm of immune system activation: various experimental observations indicate that when the number of T cells specific for a target exceeds a certain limit, or when the T cells are of particularly high affinity, they can initiate a process of activation, that results in their expression of CD40L, that in turn, can induce maturation of DC, that consequently sustain and amplify the response [4]. In the context of transplantation, the existence of a large fraction of donor-reactive T cells has been widely demonstrated (see later), favoring the latter explanation. Investigations are ongoing to better elucidate the process of initiation of the alloimmune response, and to identify potential new targets for therapies that may lead to tolerance induction. However, independent of the origin and identity of the initiating stimuli, it is clear that the process of T-cell activation inevitably passes through maturation of DC, implicating these cells as key players in the rejection response.

48.3
Direct versus Indirect Pathways of Allorecognition

A very early consequence of transplant surgery is the migration of graft-resident donor DC, as “passenger” leukocytes, to secondary lymphoid tissues of the recipient, where they present donor MHC molecules to recipient T cells via a mechanism known as the “direct pathway” of allorecognition (donor MHC + peptide X → recipient T cells). This process is characterized by a surprisingly high proportion of circulating T cells (approximately 1 out of 20) [8] that recognize allogeneic MHC molecules. In addition to donor DC, recipient DC or DC precursors, mobilized to the graft as part of the inflammatory infiltrate, acquire donor allo-Ag (by internalization of soluble MHC molecules, fragments/blebs derived from donor apoptotic or necrotic cells, or via vesicles and possibly exosomes [9] exchanged between living cells) and present MHC-derived peptides bound to self MHC molecules to recipient T cells. This phenomenon is known as the “indirect pathway” of allorecognition (self-MHC + donor MHC-derived peptide → recipient T cells). The number of T lymphocytes able to recognize allopeptides through the indirect pathway is much lower compared to the direct pathway, and is commonly considered to be at the level of classical responses to nominal Ags (<1 out of 10^5 cells) [1]. Recently, interesting observations have been published regarding the possible existence of a third pathway of allorecognition, known as the “semi-direct” pathway [10]. This pathway derives from the poorly investigated process of molecular exchange between cells. This can result, in the context of transplantation, in the acquisition by recipient APC of entire MHC molecules of donor origin that are exposed on the plasma membrane and can induce alloreactive T-cell activation via the direct pathway. The extent to which this latter process contributes to rejection is still unclear and further investigation of its role is necessary.

The relative importance of the direct and indirect pathways in the rejection response has been the subject of intense interest and also controversy for several years [11]. Classic experiments showed that there is an increase in survival of thyroid, pancreatic islet, skin, or kidney allografts when these are purged of interstitial leukocytes [1]. These observations provided a basis for the concept that the direct pathway of allorecognition is the most important component of the acute graft rejection response. More recently, it has been demonstrated that the role of the direct versus the indirect pathway depends on the type of organ/tissue transplanted, the experimental model, and the phase of rejection [4, 12]. Both pathways participate in the early phases of acute rejection, but several clinical observations indicate that T-cell responses elicited by the direct pathway decrease with time after transplantation [4]. By contrast, the role of the indirect pathway is sustained and participates in chronic rejection [4]. It has been argued that initial and repeated immune-mediated damage caused during acute rejection can predispose to chronic rejection [13]. The role ascribed to both pathways in the events leading to rejection justifies targeting both direct and indirect allorecognition in strategies to promote organ transplant tolerance.

48.4
Dendritic Cells and Tolerance Induction

Together with thymic medullary epithelial cells, thymic DC are involved in negative selection of autoreactive thymocytes, which is the major role of the thymus in establishing central tolerance [14]. There is also more recent evidence that presentation of peripherally-derived Ags by DC within secondary lymphoid tissue is crucial for the induction of T-cell tolerance to self-Ags expressed exclusively in peripheral tissues [15]. Different models have been proposed to explain the mechanism(s) by which DC may induce/maintain peripheral T-cell tolerance [16]. Thus, Steinman et al. [15] have proposed that under steady-state conditions (no inflammation), the uptake and presentation of selfAgs by immature DC expressing low cell surface levels of MHC and co-stimulatory molecules, may induce tolerance to self-reactive T cells escaping thymic selection. This prediction is based on two experimental observations: (i) binding of the T-cell receptor (TCR) on naïve T cells to MHC-peptide complexes on the APC, in the absence of or with low co-stimulation, leads to Ag-specific T-cell unresponsiveness in various experimental settings; (ii) in the healthy steady state, DC traffic continuously from the periphery to secondary lymphoid tissue transporting self-Ags [16]. However, the concept of migratory immature DC as keepers of peripheral T-cell tolerance is not entirely in agreement with the observation that lymph-borne DC, obtained by cannulation of lymphatic vessels in the steady state, exhibit signs of phenotypic maturation in all animal models investigated so far [1]. This discrepancy may be explained by the fact that a certain degree of DC maturation is required for homeostatic DC trafficking in the absence of inflammation, without affecting their ability to induce tolerance. This intermediate state has been defined as "semi-mature" by Lutz and Schuler [17]. In this "semi-mature" state, DC express levels of co-stimulatory molecules and produce pro-inflammatory cytokines insufficient to activate immune responses and instead can induce tolerance.

Interestingly, a recent observation lends further support to the concept of different maturation states of DC. Sporri et al. [18] employed an adoptive transfer system that involved the injection of TCR transgenic (tg) T cells into mixed chimeras, comprising 50% bone marrow cells with a functional TLR signaling pathway but incompatible MHC, and 50% bone marrow cells lacking Myd88 (or specific TLRs) but with MHC recognized by the tg T cells. After challenging the animals with specific Ag and TLR ligands, there was a clear indication that the "indirect" activation of Myd88$^{-/-}$ DC (through inflammatory mediators released by APC able to detect TLR ligands) induced phenotypic maturation of these cells, that could then induce proliferation of tg T cells. However, these phenotypically mature DC were not able to produce IL-12 and the proliferating T cells failed to undergo T helper (Th) cell differentiation. Even though the model was limited to the use of tg T cells and the fate of the proliferating Th0 cells was not investigated, this study underscores the excessive simplification assumed in many models of tolerance (e.g. the expression of co-stimulatory molecules associated with induction of immunity) and the neces-

sity for further investigation of specific features that distinguish tolerance-inducing and immune-stimulatory DC.

As with the model of immature-mature DC, or with the "danger" model, the results of almost all experiments investigating tolerance can also be incorporated in alternative models like the "Tunable Activation Threshold" model or the "Antigen Localization, Dose, and Time" model, both of which outline the capacity of the immune system to perceive evolving situations and respond appropriately, depending not only on the activation state of the APC, but also on the level of Ag presented and the persistence of Ag presentation [19, 20]. Moreover, it is becoming increasingly clear that the environment in which DC reside (e.g. liver versus spleen) and in which the T cell–DC interactions take place, can profoundly affect the immune response, not only in terms of Th1/Th2 skewing, but also in the decision regarding complete activation of an immune response or the induction of tolerance [21, 22].

Independent of the validity of one model versus another, different groups have shown using tg mice that constitutive, migratory MHC II$^+$ APC that transport tissue-specific Ag from the periphery silence, rather than activate, Ag-specific CD4$^+$ or CD8$^+$ T lymphocytes in secondary lymphoid tissues [23, 24]. This clearly delineates tolerance induction as an active process that is driven by the same cells that instigate the immune response, i.e. the DC.

48.5
Mechanisms underlying Dendritic-cell-induced T-cell Tolerance

The precise mechanism(s) by which immature or "semi-mature" DC induce specific T-cell tolerance to self or non-self Ags is not well understood. Current evidence suggests that more than one mechanism may be involved. One mechanism is related to the ability of DC to direct the Th1/Th2 profile of effector cells and consequently, their effector activity, through the production and secretion of specific cytokines and other molecules during activation. So-called immune deviation, or skewing of CD4 T cells toward a Th type that would be ineffective (generally toward a Th2 type), appears to be a mechanism that DC (and some parasites as well) exploit under certain experimental conditions. Thus, several groups have shown that DC can induce immune deviation in either autoimmune disease or transplant models [25]. This effect seems to be enhanced by administration, while loading the cells with specific Ags, of co-stimulation blocking agents, such as the chimeric fusion protein, cytotoxic T lymphocyte Ag 4 (CTLA4)-Ig, that blocks the B7-CD28 signaling pathway [26], or by treatment of DC in culture with an agent (i.e. IL-10) to impair their Th1-promoting activity and to increase Th2 skewing [27, 28].

Induction of a state of T-cell unresponsiveness (reversible under specific conditions) defined as anergy [29] and/or elimination of reactive T cells by stimulating their apoptosis, seem to be strategies used largely by the immune system to control potentially deleterious autoreactive responses. Many studies have shown that DC whose allostimulatory function is impaired, either by incomplete maturation, se-

lective blockade of surface B7 co-stimulatory molecules, the influence of specific cytokines [e.g. IL-10 or transforming growth factor-β (TGF-β)], or genetic engineering (to express viral IL-10, CTLA4-Ig, or CD178 = FasL), can induce alloAg-specific T-cell hyporesponsiveness (anergy) or apoptosis *in vitro*, and suppress immune reactivity [25, 30]. In particular, considering Ag-specific T-cell deletion as a robust tolerance-inducing strategy, various and successful attempts have been made to enhance this process through over-expression of molecules associated with the induction of apoptosis, i.e. CD178 [31, 32], nitric oxide (NO) [33, 34] or the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) [35], rendering DC able to subvert T-cell responses by promoting activation-induced cell death. Blockade of the B7/CD28 pathway by CTLA4Ig significantly increases myeloid DC-induced apoptosis of allo-activated T cells [32]. This appears to be mediated, at least in part, via the Fas pathway. On the other hand, recent investigations indicate that ligation of B7 molecules by CTLA4-Ig induces upregulation of IDO in DC, causing a local reduction in tryptophan availability and production of pro-apoptotic kynurenines that decrease T-cell clonal expansion and enhance their deletion [35].

Evidence has also emerged that DC can promote the induction and expansion of T cells with regulatory activity and even control their function; these properties are under active investigation by a large number of laboratories, due to the potential benefit of DC-based therapy for long-term, Ag-specific unresponsiveness (Table 48.1). An example of the ability of DC to expand regulatory cells is given by $CD86^+$ DC, that stimulate the expansion of regulatory T cells (Treg) and, when loaded with defined Ags, drive the expansion of Ag-specific Treg [36]. Various Treg cell populations with suppressor activity have been described. They can be divided roughly into two families: "induced" (which derive from normal T cells stimulated under specific conditions) and "naturally arising" (that appear to be committed to regulation since their development). T-regulatory-1 (Tr1) and Th3 cells are induced following T-cell activation in the presence of IL-10 and/or TGF-β and are characterized by high production of IL-10 and TGF-β respectively. $CD4^+CD25^+$ T cells arise spontaneously during ontogeny (the majority of them, if not all, are generated in the thymus) and are present in the periphery of normal mice, where they constantly control autoreactive responses of self-reactive T cells that have escaped thymic selection. Other Treg cell subtypes have been described, including $CD8^+CD28^-$, $CD3^+CD4^-CD8^-$, $CD8^+CD25^+$, γδ and NKT cells [37] but, with the exception of $CD8^+CD28^-$ cells (see later), they remain poorly characterized in comparison to $CD4^+CD25^+$ cells. Comprehension of the molecular interactions underlying generation/expansion of Treg cells by DC will allow the identification of key targets that could be exploited in new therapeutic approaches using improved "tolerogenic" DC. The involvement of T cells with regulatory activity has been shown clearly in various animal models of transplant acceptance [38]. Further interest in generation of Treg cells has recently been paralleled by the emerging concept that interaction between tolerogenic DC (also termed regulatory DC) and Treg cells may result in a bi-directional stimulation, generating an inhibitory feedback loop that can create a self-sustained state of tolerance [39] (Table 48.2). This feedback has been described in a mouse model following a short course of anti-CD45RB mAb, together with the

Tab. 48.1 DC-induced generation/expansion of T regulatory cells.

Strategy	*Result(s)*	*Species/ System*	*Ref.*
Stimulation of $CD4^+CD25^+$ regulatory T cells with high doses of antigen-loaded myeloid DC	Expansion of $CD4^+CD25^+$ T cells (IL-2-dependent) that maintain their suppressive abilities	Mouse, *in vitro*	112
Co-culture of naïve $CD4^+CD25^-$ T cells with CpG-stimulated allogeneic plasmacytoid DC	Induction of hyporesponsive $CD4^+CD25^+$ T cells that inhibit proliferation of autologous or allogeneic naive $CD4^+$ T cells, in an Ag-non-specific manner	Mouse, *in vitro*	103
Use of OVA peptide-pulsed $CD11c^{lo}CD45RB^{hi}$ DC – present in the spleen and lymph nodes of normal mice	Induction of tolerance through the differentiation of Tr1 cells	Mouse, *in vitro* and *in vivo*	113
Injection of OVA coupled to anti-DEC-205 monoclonal antibody (that targets DC)	Identification of $CD25^+/CD152^+$ T cells that suppress proliferation of IL-2 production by effector $CD4^+$ T cells	Mouse, *in vivo*	114
Injection of bone marrow-derived 'regulatory' DC (treated with IL-10, TGF-β, and LPS)	Recovery of increased numbers of $CD4^+CD25^+CD152^+$ T cells with suppressive properties	Mouse, *in vivo*	56
Injection of dexamethasone-treated DC co-expressing donor and recipient MHC	Inhibition of indirect T cell alloreactivity due to expansion of $CD4^+CD25^+$ T cells with regulatory properties	Rat, *in vivo*	62
Repetitive T-cell stimulation with immature, allogeneic monocytoid DC	Generation of IL-10 producing, non-proliferating $CD4^+$ T cells with regulatory properties	Human, *in vitro*	40
Injection of immature, monocytoid DC pulsed with influenza matrix peptide (MP)	$CD8^+$ T cells obtained 7 days after injection suppress MP-specific effectors	Human, *in vivo*	41

* ovalbumin

deoxyspergualin (DSG) analog LF15-0195, that induced long-term acceptance of the transplant. From these tolerant recipients, Min et al. [39] isolated tolerogenic DC, able to generate/expand $CD4^+CD25^+$ Treg cells from naïve T cells *in vitro*. In parallel, $CD4^+CD25^+$ T cells from the same graft recipients induced tolerogenic DC (able to prime T cells with suppressive properties) when co-cultured with bone marrow progenitors.

Most data that support a role of DC in controlling immune responses have been generated in small animal models, but there are many clear indications from human studies of the regulatory potential of DC-based therapeutic strategies. Human

Tab. 48.2 Outcome of interaction between T regulatory cells and DC.

Strategy	*Result(s)*	*Species/ System*	*Ref.*
Anergized $CD4^+$ T cell clones co-cultured with bone marrow-derived DC	Inhibition of immunogenicity of DC, that can then induce anergy in responsive T cells	Mouse, *in vitro*	115
Co-culture of bone marrow derived DC with $CD4^+CD25^+$ regulatory T cells	Induction of immunosuppressive tryptophan catabolism in DC	Mouse, *in vitro*	116
Co-culture of splenic DC or $CD4^+CD25^+$ T cells from tolerant transplant recipients with naïve T cells or DC progenitors, respectively	Isolated DC induce generation/ expansion of $CD4^+CD25^+$ Treg. Isolated Treg generate DC with tolerogenic-DC phenotype	Mouse, *ex vivo*	39
Co-culture of monocyte-derived DC with $CD8^+CD28^-$ alloAg-specific T suppressor cells	Upregulation of ILT3 and ILT4 on DC, that can then induce Ag-specific unresponsiveness in $CD4^+$ T cells	Human, *in vitro*	42

$CD4^+$ T cells with characteristics of Treg cells (low proliferative capacity, secretion of IL-10, and ability to inhibit alloAg-specific proliferation of other T cells) can be generated *in vitro* following repetitive stimulation of naïve $CD4^+$ T cells with allogeneic immature DC [40]. Furthermore, in human volunteers, immature autologous monocyte-derived DC, pulsed with the human leukocyte Ag (HLA)-A*0201-restricted influenza matrix peptide (MP), induced specific inhibition of MP-specific $CD8^+$ cytotoxic T lymphocytes (CTL) and induced IL-10-secreting $CD8^+$ T lymphocytes [41]. Moreover, Chang et al. [42] were able to generate tolerogenic DC by co-culturing immature DC with allospecific $CD8^+CD28^-$ T suppressor cells or $CD4^+CD25^+CD45RO^+$ Treg cells in a human *in vitro* model system. These tolerogenic DC were characterized by increased surface expression of the inhibitory molecules immunoglobulin-like transcript-3 (ILT3) and ILT4 and could inhibit the capacity of alloreactive CD4 T cells to proliferate, further converting them to Treg cells that could continue the cascade by tolerizing other APCs.

These results constitute proof of the principle of DC tolerogenicity in human systems that is already driving new investigations in DC-based therapy related to the importance of tolerogenic DC–T cell interaction in regulation of alloimmune responses. These investigations are also aimed at delineating the various parameters that may influence this interaction (e.g. the route and frequency of *in vivo* DC administration, the temporal relationship between their administration and that of Ag, the number of cells injected, the amount and physical condition of Ag presented, the nature of any immunosuppressive drug therapy, and the source, type and maturation state of the DC).

48.6
Dendritic Cells and the Control of Organ Transplant Outcome

At face value, the contribution of DC to transplant rejection may seem quite straightforward within the framework of direct and indirect allorecognition. However, in apparent contradiction to the classical "passenger leukocyte" experiments, several groups have reported that depletion of donor bone marrow (BM)-derived cells prevents the induction of transplantation tolerance [4, 43]. For example, depletion of passenger leukocytes from rat donor heart allografts reversed the beneficial effects of donor-specific blood transfusion. Furthermore, tolerance was re-established if donor-type DC were co-transferred at the time of transplantation of APC-depleted cardiac grafts [43]. An interesting aspect of the role of donor DC in transplant immunity/tolerance concerns the phenomenon of donor hematopoietic cell "microchimerism", as described by Starzl et al. [44, 45]. Chimerism is the existence of cells from different genetic backgrounds in a single organism; in transplantation, it refers to the presence of donor-specific cells, usually DC, in the recipient. Starzl et al. [44] detected donor hematopoietic cells in lymphoid and nonlymphoid tissues of long-surviving human organ allograft recipients, including patients off all anti-rejection therapy. It was proposed that the ability of an organ to be tolerogenic, in the absence or presence of effective immunosuppression, was dependent on its passenger leukocyte, and not its parenchymal cell component. In support of this view, the radiosensitive passenger leukocyte population of renal allografts has been implicated in the induction of tolerance to contemporaneous heart grafts in a miniature swine model [46]. Other indirect evidence for a role of DC in the control of transplant outcome derives from impressive results obtained in a rhesus macaque renal allograft model. Using a combination of peri-transplant treatment with a T-cell-depleting agent (anti-CD3 immunotoxin) and a 15-day course of DSG, a NF-kB inhibitor that suppresses DC maturation and pro-inflammatory cytokine production [47], 87% of the recipients showed allograft survival with no rejection, without the need for continued immunosuppressive treatment. The efficacy of this treatment correlated with significant reduction of mature DC in recipient lymph nodes, together with a coincident reduction in lymph node T-cell mass. Even if some issues remain unresolved regarding microchimerism, like the uncertainty whether persistence of donor leukocytes in tolerant patients represents cause or effect [4], the general understanding of a role of DC in peripheral tolerance, together with the development of techniques to expand large numbers of DC *in vitro*, have opened up the possibility of generating DC with tolerogenic/regulatory properties for therapeutic application.

Various experimental techniques have been used to generate DC progenitors or DC precursors with tolerogenic potential. These can be grouped under three technologic approaches: (i) specific culture conditions, (ii) pharmacological manipulation, and (iii) genetic engineering.

48.6.1 Dendritic Cell Manipulation for Tolerance Induction: Specific Culture Conditions

In line with the classic model of T-cell stimulation [48], which underscores the significance of interaction between "immature" DC (that are not simply ignored) and T cells as an active process underlying tolerance induction, the first attempt to produce tolerogenic DC was the generation of immature myeloid DC (MHC^+, $CD80^{lo/-}$, $CD86^{lo/-}$) in culture [49]. This early work showed that such immature myeloid DC could induce alloAg-specific T-cell hyporesponsiveness *in vitro*. When injected into prospective recipients of vascularized heart allografts, the same cells induced significant donor-specific prolongation of graft survival (third party grafts were rapidly rejected) in the absence of anti-rejection therapy [39]. Subsequent studies have shown that, if administered before, during, or even after transplantation, immature donor-derived DC can prolong allograft (including skin graft) survival. In some instances, indefinite, donor-specific graft survival has been achieved [50–53]. A potential drawback of this approach is the possibility that, following injection, a fraction of the immature donor DC may differentiate *in vivo* into mature APCs, with ability to stimulate an anti-donor response and accelerate graft rejection. In an effort to overcome this potential problem, some investigators have combined administration of immature, donor-derived DC with a short course of anti-CD40 ligand (anti-CD154) mAb, to avoid any potential stimulatory effects/maturation of the injected DC in the recipient through CD40-CD154 interaction (but there is also recent evidence of T-cell depleting effects of this mAb), and obtained striking enhancement of graft survival [54]. Administration of LPS-, TNF-α-, and CD40-maturation resistant donor-derived DC has proven an alternative means to avoid *in vivo* DC maturation, with indefinite (>100 days) prolongation of heart allograft survival in nonimmunosuppressed mice [55]. Recently, Sato et al. [56] found that mouse BM-derived DC generated in IL-10 + TGF-β in addition to GM-CSF, then stimulated with LPS acquired regulatory functions; if generated from host BM, a single injection (two days after transplant) of these "alternatively-activated" DC protected mice from lethal, allogeneic BM-induced graft-versus-host disease. Furthermore, these DC impaired dramatically the anti-recipient response of allogeneic CD4 and CD8 T cells through induction of Ag-specific $CD4^+CD25^+CD152^+$ Treg cells in the graft recipients. A similar regulatory effect (induction of anergic and Treg cells) has been reported for human monocyte-derived DC cultured with IL-10+TGF-β (in addition to GM-CSF and IL-4) [57]. Similarly, using human DC treated with IL-10, Steinbrink et al. [58] were able to induce CD4 and CD8 anergic T cells displaying allo-Ag specific suppressor activity.

48.6.2 Dendritic Cell Manipulation for Tolerance Induction: Pharmacological Manipulation

In an effort to obtain DC with a stable, immature phenotype, or with impaired ability to synthesize Th1-driving cytokines (i.e. IL-12p70), DC have been treated with diverse pharmacological agents. The spectrum of molecules investigated includes:

aspirin, cyclic adenosine monophosphate (cAMP) inducers (prostaglandin E2, histamine, β2 agonists, neuropeptides), the vitamin D3 metabolite 1a,25-(OH)2D3 and its analogs, glucosamine, the antioxidant *N*-acetyl-L-cysteine, and immunosuppressive drugs (corticosteroids, cyclosporine, rapamycin, DSG, and mycophenolate mofetil). Each of these molecules prevents DC activation/maturation, or impairs the capacity of DC to produce bioactive IL-12p70 *in vitro* and *in vivo* [59]. The mechanism(s) of action of many of these compounds is known. Thus, *N*-acetyl-L-cysteine, aspirin, 1a,25-(OH)2D3, corticosteroids, cyclosporine, and DSG prevent nuclear translocation of specific members of the NFκB family of transcription factors required for DC differentiation. In many instances, these pharmacologically-manipulated DC became resistant to maturation-inducing stimuli, and their interaction with T cells causes Ag-specific unresponsiveness. In mice, treatment with donor-derived DC generated *in vitro* in the presence of the active form of vitamin D3 (1a,25-(OH)2D3) in combination with mycophenolate mofetil, induced tolerance to fully-mismatched pancreatic islet allografts [60], with generation/amplification of Treg cells (able to confer protection against islet rejection in naïve animals). Treatment of DC with immunosuppressive drugs, in particular dexamethasone, which arrests their differentiation/maturation [61] offers potential for development of "negative cellular vaccines" for immunotherapy. An encouraging and clinically-relevant application of these cells has been described by Mirenda et al. [62]. These authors have shown that pre-treatment of rats (7 days before transplant) with dexamethasone-treated DC (obtained from F1 donors) co-expressing donor and recipient MHC molecules, together with a single dose of CTLA4-Ig (one day later), lead to indefinite (>100 day) kidney allograft survival after a short post-operative course of cyclosporine (to inhibit the early direct pathway response). There appeared to be no evidence of chronic rejection in the grafts. This striking therapeutic effect was associated with the presence and function of indirect pathway Treg cells [62]. Similarly, very promising results have been reported recently using rapamycin, which impairs the generation of DC (at clinically relevant doses), suppresses DC maturation and IL-12p70 production, and decreases the T-cell stimulatory ability of DC *in vivo* [63]. Adoptive transfer of DC from rapamycin-treated donors induced allo-Ag-specific T-cell hyporesponsiveness [63]. In further experiments, rapamycin-treated, donor alloAg-pulsed host-derived DC were found to prolong heart graft survival, an effect that was enhanced by repeated infusion of the cells. Collectively, these data from experimental models confirm the validity and importance of pharmacological modifications aimed at modulating the Ag-presenting function of DC to promote a robust form of tolerance.

48.6.3
Dendritic Cell Manipulation for Tolerance Induction: Genetic Engineering

Recent advances in gene transfer technology have resulted in enhancement/ stabilization of the tolerogenic potential of DC following their genetic modification to express "immunosuppressive" molecules that can: (i) inhibit or block cell-surface costimulatory molecule expression (viral or mammalian IL-10, TGF-β, CTLA4-Ig)

and skew the Ag-specific T-cell response towards Th2 predominance, (ii) prevent proliferation of allogeneic T cells (IDO), (iii) induce and maintain T-cell anergy (B7-H1), and (iv) promote deletion (through apoptosis induction) of Ag-specific T cells (CD178; TNF-related apoptosis-inducing ligand [TRAIL]) [30]. Various transplantation models have been used to test the feasibility of this approach. Multiple intraperitoneal injections of DC transduced to express human FasL markedly extended mouse-vascularized heart allograft survival [64]. Mouse DC line cells expressing CTLA4-Ig were used to prolong pancreatic islet allograft survival [65]. Not all the genetic manipulations gave positive results. An example is the pretreatment of heart allograft recipients with IL-4-transduced DC which resulted in exacerbated rejection [66]. This observation, that contrasts with the striking therapeutic efficacy of IL-4-transduced, donor-derived DC in murine collagen-induced arthritis, probably reflects a different requirement/involvement of Th1/Th2 responses that varies according to the type of immune response under study [67]. It is important to consider that, to date, there have been no reports of robust, donor-specific tolerance being achieved across MHC barriers using genetically-modified, donor-derived DC alone. This could be ascribed to various factors: (i) unsustained immaturity of the genetically-modified DC following their administration *in vivo* (as with the pioneering work with immature DC); (ii) inappropriate or inadequate numbers of administered DC; (iii) sub-optimal route or frequency of DC injection; (iv) altered transgene expression *in vivo*; (v) low efficiency of transduction of administered DC; (vi) adverse effect (immunostimulatory) of transfection vectors. Alternative approaches have consequently been tested in order to improve the efficacy of genetic manipulation of DC. Portal venous infusion of a mixture (1:1) of donor-derived DC transduced with either TGFβ1 or IL-10 resulted in significant prolongation of mouse renal allograft survival. With regard to the issue of maturation after injection, an important recent finding is the possibility of using NFκB-specific "decoy" oligodeoxyribonucleotides (ODNs) that stably inhibit DC maturation, even in response to maturation-inducing stimuli, without affecting transgene expression. A single, pre-transplant intravenous infusion of NFκB ODN-treated, CTLA4-Ig-transduced donor DC markedly prolonged fully MHC-mismatched vascularized heart allograft survival, with 40% of the animals exhibiting long-term (>100 day) graft survival [68]. Conceptually, appropriate trafficking of DC expressing these molecules to secondary lymphoid tissues, where Ag presentation and Ag-specific T-cell responses are initiated, should minimize potential undesired side effects that may be associated with systemic administration of the same molecules. Finding an optimal means of optimizing the migratory pattern of the injected DC (e.g. by co-transfer of appropriate chemokine receptor genes) is likely to increase the efficacy of the genetic manipulation approach.

48.6.4
Use of Specific Dendritic Cell Subsets for Tolerance Induction

So far, we have referred generically to DC without any reference to specific subsets. DC constitute a heterogeneous population. Many different subsets have been char-

acterized in mice and two in humans (monocyte-derived "DC1" and plasmacytoid "DC2"). A detailed description of these subsets can be found in other chapters in this book. With regard to DC therapy for transplant tolerance, considerable effort is now focused on the potential of distinct DC subsets. All of the approaches described above have been tested using classic myeloid DC. In mice, these are $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ and are generally propagated from BM cells. Human myeloid DC are usually propagated from blood monocytes.

Considerable initial enthusiasm followed the observation that murine $CD8\alpha^+$ ("lymphoid-related") DC induced weak proliferation of T cells, mainly due to FasL-mediated apoptosis of $CD4^+$ T lymphocytes and limited IL-2 secretion by $CD8^+$ T cells. This led to the concept of the existence of a specific DC subset whose main function was induction of tolerance. This concept has been seriously challenged by several observations showing that $CD8\alpha^+$ DC can secrete high levels of IL-12p70, induce generation of Th1 cells, and stimulate cytotoxic T cells [69–71]. These observations underscore the significance of DC "plasticity", defined as the ability of DC to induce, modulate, or prevent an immune response, depending on the stimuli received from the environment and on their interaction with other cells of the innate and adaptive immune responses. However, this has not impeded the investigation of $CD8\alpha^+$ DC as tools to induce tolerance. Intravenous administration of donor-derived splenic $CD8\alpha^+$ DC prolonged the survival of vascularized heart allografts in a mouse model. Interestingly, this effect was achieved whether or not the $CD8\alpha^+$ DC were immature, or had undergone *in vitro* maturation [50]. Further attention to the use and successful manipulation of $CD8\alpha^+$ DC derives from the physiologically high expression of IDO in this subpopulation [72, 73], together with the suppression of allogeneic T-cell proliferation *in vitro* by DC expressing IDO as a transgene [74]. This observation is rendered more significant considering that, even if a $CD8^+$ human counterpart of DC has not been defined, it has been reported recently that the expression of IDO by a subpopulation of human DC (characterized by surface expression of CD123 and CCR6) may have important regulatory implications [75].

Considerable expectations have grown around the most recently discovered DC subset defined as plasmacytoid (p)DC, identified initially in humans. In mice, a recently-identified cell population resembling human pDC produces large amounts of type I IFN upon viral stimulation, and differentiates into mature DC following CD40 ligation. Unlike human pDC however, murine pDC are surface $CD11c^{lo}$ and produce IL-12. Preliminary data indicate that a single pre-transplant infusion of highly-purified, freshly-isolated donor pre-pDC from mouse secondary lymphoid tissue markedly prolongs vascularized organ allograft survival [51]. Recently, precursors of plasmacytoid DC (pre-pDC) have been identified as key components of the $CD8^+/TCR^-$ BM cell population that facilitates hematopoietic stem cell (HSC) engraftment and induce skin graft tolerance across MHC barriers, without causing GVHD [76], lending further support to their potential for tolerance induction. The identification of pre-pDC in the circulation of rhesus monkeys, mobilized *in vivo* by Flt3L or G-CSF, suggests that comparatively large quantities of these cells can be prepared for *in vivo* testing of their tolerogenicity [77]. With regard to the potential of the human pDC subset, it has been reported [78] that immature pDC, fresh-

ly isolated from human peripheral blood, can induce Ag-specific anergy in $CD4^+$ T-cell lines. This phenomenon may involve the inhibitory receptors ILT3 and 4, expressed on the surface of immature pDC [79]. Moreover, priming of human naïve $CD8^+$ T cells *in vitro* with allogeneic, CD40L-activated pDC induces the differentiation of IL-10^{hi}IFN-γ^{lo} CD8 Treg cells that are capable of bystander suppression of CD8 T-cell proliferation [80]. In comparison to myeloid DC, strategies employing plasmacytoid DC could take advantage of retention of the cell's tolerogenic potential following exposure to maturation stimuli (as shown *in vitro* by CD40L-activated pDC) that constitutes a concern in strategies involving use of immature myeloid DC for tolerance induction.

48.6.5
Dendritic Cell Therapy: Targeting the Indirect Pathway

The encouraging and in some instances impressive results obtained using various donor-derived DC to prevent rejection in small animal models seem to contrast with the concept of rejection determined by both direct and indirect pathways of T-cell allorecognition, and the need to inhibit both pathways to adequately control rejection. Two factors probably account for these results. First, almost all the successful tolerogenic strategies have used DC in combination with other treatments to subdue the immune response and promote a "steady state". Inevitably, these treatments (e.g. anti-CD154 mAb, or T-cell-depleting strategies) also affect the indirect pathway, diminishing its contribution and rendering it less evident. Moreover, it has been shown that donor-derived DC can transfer allogeneic MHC molecules to recipient DC *in vivo* [4]. These recipient APC can then present donor-derived MHC peptides in the groove of self MHC molecules. Thus, it has been argued that administration of donor DC may also influence the indirect pathway of allorecognition. Given that the role of the direct pathway diminishes with time after transplantation, while that of the indirect pathway appears to be sustained and participates in chronic rejection, attempts have been made to target the indirect pathway via DC therapy.

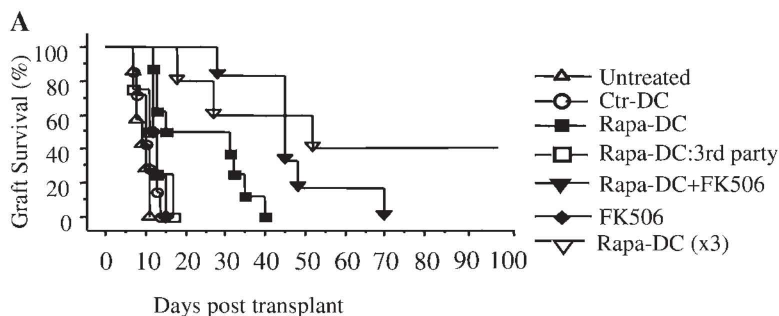
Initial efforts were performed in a rat model. Recipient DC (BM-derived or thymic) pulsed *ex vivo* with immunodominant donor MHC I-derived allopeptides, were injected into the thymus of recipients, 7 days before transplant. This led to permanent survival of cardiac or pancreatic islet allografts when administered with antilymphocyte serum [81, 82]. Furthermore, robust and permanent tolerance was obtained in this model, as demonstrated by the absence of rejection after challenge with a second, donor-type heart graft. A similar effect was observed when recipient DC, pulsed with donor allopeptides, were administered intravenously; this is a more feasible route in relation to possible clinical application [83, 84]. An interesting approach to presentation of donor MHC antigens by recipient DC is the transduction of recipient-derived DC with a vector encoding donor-type MHC class I gene and the injection of these cells one month before transplant. Combination of this treatment with anti-CD4 mAb prolonged the survival of fully allogeneic murine hearts grafts significantly [85]. These findings suggest that genetically-modi-

fied, autologous DC may prove useful for delivery of donor allo-Ags for the promotion of transplant tolerance. This approach still has to resolve how to present all relevant and necessary donor MHC molecules in a feasible strategy.

Currently, there are two promising approaches to pulsing of recipient DC with immunodominant MHC-derived peptides: (i) *in vivo* targeting of recipient DC, and (ii) the use of pharmacologically-modified DC loaded *ex vivo*. Both approaches are aimed at maintaining/improving the tolerogenic status of recipient DC with respect to presentation of allo-Ag. Regarding *in vivo* targeting of recipient DC, two strategies have shown interesting results. The first strategy concerns the use of mAbs to target molecules expressed specifically/mainly by immature DC. The most significant results have been obtained with anti-CD205 mAb. Following systemic delivery of ovalbumin (OVA) coupled to anti-CD205 mAb in a TCR tg mouse model, recipient DC process and cross-present an OVA-derived peptide loaded in MHC I molecules to TCR tg $CD8^+$ T cells. Presentation of Ag in this manner induces weak stimulation of the Ag-specific $CD8^+$ T cells, followed by their deletion and the establishment of $CD8^+$ T-cell-specific tolerance [86]. The second strategy employs intravenous administration of apoptotic donor MHC^+ cells. Considerable interest is focused on the tolerogenic properties of apoptotic cells (see Chapter 29). With regard to use of apoptotic bodies to induce transplant tolerance, encouraging results were obtained by intravenous administration of donor MHC^+ early apoptotic cells (splenocytes), 7 days before transplant in a mouse model. This resulted in significant prolongation of vascularized heart graft survival in the absence of immunosuppressive therapy [1]. The therapeutic effect correlated with internalization of the injected apoptotic bodies by host DC, as adoptive transfer of splenic DC from mice pretreated with the allogeneic apoptotic cells into naïve recipients prolonged graft survival.

The second approach to improving the beneficial effects of loading recipient DC with alloAgs relates to the *ex vivo* treatment of DC with an immunosuppressive drug(s) in order to generate tolerogenic DC that can be loaded with donor Ags by simple exposure in culture to lysates of donor-derived cells. The underlying principle is that these DC will maintain their tolerogenic properties when injected into graft recipients. A promising example concerns pre-operative infusion of mouse organ allograft recipients with rapamycin-treated, recipient-derived DC pulsed with donor spleen cell lysate, a treatment that significantly prolongs heart graft survival in a donor-specific manner (Fig. 48.1) [87]. In view of the finding that simple injection of *in vitro*-generated, recipient-derived DC (without donor Ag) can prolong (rat) organ graft survival [88], but in an Ag-non-specific manner (and by an unknown mechanism that seems to relate to nitric oxide metabolism), both targeting of DC *in vivo* via injection of donor apoptotic cells, or loading of pharmacologically-modified host DC with donor cell lysate, are of particular promise. These approaches allow the problem of tolerizing the immune system against the entire complex repertoire of immuno-dominant donor Ag to be overcome, without having to know every Ag specificity and, importantly, without having to work with each Ag individually, as envisaged with gene therapy or Ab-coupled approaches.

A



B

Group	n	Graft Survival (days)	MST (days)
1 Untreated	7	7, 8(x2), 9, 10, 11(x2)	9.1
2 Ctr-DC	7	7, 8, 10(x2), 11, 13, 14	10.4
3 Rapa-DC	8	12, 13(x2), 15, 31, 32, 35, 40	23.8[a]
4 Rapa-DC+IL-2	4	6, 6, 6, 8	6.5
5 Rapa-DC:3rd party	4	7, 11, 13, 17	12
6 Rapa-DC+FK506	6	28, 45(x3), 48, 70	46.8[b,c]
7 FK506	4	12(x2), 13, 15	13
8 Ctr-DC (x3)	4	9, 9, 11, 15	11
9 Rapa-DC (x3)	5	18, 27, 52, >100 (x2)	>59[d,e]

^{a}P<0.0001 compared with group 1; ^{b}P=0.0005 compared with group 1 and ^{c}P=0.0029 compared with group 3; ^{d}P=0.0014 compared with group 1 and ^{e}P=0.0464 compared with group 3
MST: mean survival time

Fig. 48.1 AlloAg-pulsed Rapa-DC prolong heart graft survival. AlloAg-pulsed, control (Ctr)- or Rapa-DC of host strain origin were injected i.v. into syngeneic C3H mice, 7 days before transplantation with B10 heart grafts. FK506 (1 mg/kg/day) was administered i.m. for 10 days from the time of transplant, where indicated. Untreated, third party (BALB/c) lysate-pulsed Rapa-DC-treated (Rapa-DC:3rd party) and FK506-treated mice were used as control recipients. Alternatively, the alloAg-pulsed Rapa-DC were infused i.v. ×3 (days –10, –3 and 0) into otherwise unmodified graft recipients. ^{a}P<0.0001, ^{b}P = 0.0005 and ^{d}P = 0.0014 vs. untreated mice; ^{c}P = 0.0029 and ^{e}P = 0.0464 vs. Rapa-DC-treated mice, analyzed by log-rank test. (Reproduced with permission from Taner T et al., *Am J Transplantation*, **2005**;5:228–236.)

48.6.6
Dendritic Cells and the Treatment of Chronic Rejection

DC therapy appears to be effective (in experimental small animal models) in ameliorating chronic rejection (transplant vascular sclerosis) that current immunosuppressive drugs fail to prevent. Unfortunately, there is as yet rather limited information about the extent of its potential and the mechanism(s) by which DC could be used in this regard. A recent report shows no signs of chronic rejection (by histo-

logical examination) in fully allogeneic cardiac grafts, 100 days after transplantation in recipients pre-treated with donor splenic DC plus blocking anti-CD154 mAb [89]. Moreover, Wang et al. [90] have demonstrated that peri-transplant administration of purified donor immature splenic DC, in combination with blocking anti-CD154 mAb, strongly inhibits the development of intimal thickening, fibrosis, and proliferation of α-smooth muscle actin$^+$ cells in a murine aortic allograft model. More detailed investigations are necessary, especially in large outbred animal models, but these early findings suggest that strategies aimed at preventing chronic rejection should involve the testing of DC-based therapies.

48.7 Dendritic Cells and Cellular Markers of Transplant Tolerance

It is becoming increasingly evident that different approaches using DC to promote tolerance may be feasible. Unfortunately, testing of these strategies in humans and progress towards clinical trials for transplant therapy, are hampered by the absence of reliable diagnostic markers that can be used to precisely monitor the anti-donor immune response of the recipient, and to determine when a state of stable tolerance has been obtained. In fact, the absence of these markers currently limits the investigation of drug withdrawal strategies, as the risk of losing the function of a (life-supporting) transplanted organ remains too high and cannot be justified. Studies are ongoing to identify such markers. Interesting results have been obtained recently in a study aimed at correlating the frequency of specific peripheral blood mononuclear populations with the state of operational tolerance in pediatric liver transplant patients [91]. The most significant findings were a reduction in the NK cell (CD3$^-$CD56$^+$) population and a concomitant increase in the B cell (CD19$^+$) population in the tolerant group. More interestingly, a significant increase was also observed in the frequency of CD4$^+$CD25^{hi} T cells in tolerant patients. A further difference was an increase in the number of γδ T cells of the Vδ1 type, which have been reported to become a dominant population during normal, and not abortive, pregnancy. Apart from speculation regarding the CD4$^+$CD25^{hi} population, whose increase could be attributed to an expansion of cells responsible for controlling the alloresponse (this has yet to be demonstrated), the mechanistic significance of these findings are unclear. A similar study [92] identified a significant increase in the proportion of NKT cells (which may represent a regulatory population) in rejection-free, cadaveric kidney allograft recipients maintained on minimal immunosuppression. Interestingly, recent studies have outlined the possibility of using DC subsets as a monitoring tool. Mazariegos et al. [93] have observed significantly higher incidences of pre-pDC relative to monocytoid DC precursor in blood of clinically tolerant liver allograft recipients and in patients on low dose anti-rejection therapy successfully undergoing drug weaning, compared with those on maintenance immunosuppression. Furthermore, this ratio was not affected by the type or extent of immunosuppressant received by the patient [94]. Considering that reductions in circulating pDC2 have been associated with acute and chronic GVHD af-

ter stem cell transplantation [95], and that comparatively high levels of pDC2 are linked with poor prognosis in childhood cancer [96], there is sufficient evidence to support further studies of the use of pDC2 levels as a correlate/predictor of the tolerant state. It will be particularly interesting to ascertain whether a correlation may exist between the observed increases in pre-pDC and in those of $CD4^{+}CD25^{hi}$ T cells in tolerant liver graft recipients, as it can be speculated that dynamic interactions may be ongoing between these populations. Furthermore, monitoring the phenotype of "tolerogenic" DC (e.g. cells expressing ILT3 and ILT4) may provide significative insight into this field as analyses that focus on frequencies of cells with tolerogenic features such as these may be more predictive.

48.8 Toward Clinical Use of Dendritic-cell-based Therapies for Tolerance Induction: Critical Considerations and Future Challenges

After almost 30 years of investigation, the role of DC as initiators and as regulators of the immune responses has been established. The time has now arrived for optimization of DC-based therapies for the induction of transplant tolerance and its clinical application. Several parameters need to be carefully evaluated: definition of reproducible DC culture conditions, standardization of DC-conditioning and Ag-loading procedures, determination of optimal timing, site, and frequency of injections. All these variables will need to be evaluated extensively in human subjects in order to develop safe and effective approaches. Pharmacological manipulation of donor's and/or recipient's DC seems to be the most promising strategy, but all the aforementioned parameters still have to be optimized, together with the employment of improved immunosuppressive drugs (with more effective and less toxic effects). Furthermore, strong feedback from basic research on DC biology will be vital to improve approaches currently available and to develop new ones. As mentioned before, new manipulation strategies may be required, e.g. to promote/enhance specific localization of these cells in appropriate T-cell areas after their injection. It has been shown recently in mice that donor thymic DC injected intravenously home to the thymus and, in combination with partial T-cell depletion, prolong graft survival [97]. If cultured DC can be manipulated to increase their migration to areas relevant to tolerance induction, then more powerful and feasible strategies will become available. This is just an example of the positive drawback that can be obtained by a more detailed knowledge of DC biology. There are many different areas that will have a significant impact on the effectiveness of DC therapies for tolerance induction.

48.8.1 Dendritic Cell–T Cell Interaction

Significant advances are likely to derive from more detailed understanding of the molecular dialogue between DC and T cells. It has been reported recently that DC

can determine the Th1/Th2 orientation of the immune response not only via secreted soluble factors, but also by modifying the ratio between different Notch ligands expressed on the cell surface [98]. This adds specific surface molecules to the list of DC-derived products (mainly cytokines) that can influence the type of T-cell response and, more importantly, adds new potential targets for DC manipulation. Additional targets include recently-identified co-regulatory (co-stimulatory and co-inhibitory) molecules. A relevant field, that has moved from the simple dichotomy of CD28 versus CTLA4 interactions with CD80 and CD86, to a more complex picture, now involves many different molecules that include: CD70, OX40L, 4-1BB, and ICOSL (co-stimulatory molecules) and B7-H1, B7-DC, B7-H3 and B7-H4 ("co-regulatory" molecules), some of which possess both stimulatory and inhibitory properties, depending on the counter-receptor that they engage. All these molecules can be expressed at different levels on the surface of DC (during the maturation process) and their relative expression ratios are likely to determine the nature of T-cell responses. This list of potential targets continues to grow. Thus, Sedy et al. [99] have recently identified herpes virus entry mediator (HVEM) as the unique ligand of the inhibitory receptor B and T lymphocyte attenuator (BTLA) expressed on T and B cells that was thought to interact with B7-H4 (a B7-family member with co-inhibitory function). The potential beneficial effect of targeting these new pathways to allow control of the alloimmune response has been indicated in recent investigations [100, 101]. However, some negative results have also been reported (rejection of organ expressing supposed inhibitory ligands) [8], indicating that further characterization of the roles of these new pathways is needed.

48.8.2
Dendritic Cells and Treg: a Complex Inter-relationship

A fuller understanding of the interactions between DC and Treg cells is likely also to aid in the design of new therapeutic strategies. It has been shown recently that DC can expand previously existing $CD4^+CD25^+$ T cells (Table 48.1). In particular, use of DC loaded with a nominal Ag (the target of diabetogenic tg T cells in an animal model) allowed the expansion of Treg cells able to robustly inhibit the onset of type-1 diabetes [36]. Much work is ongoing to define the conditions for generation of new Treg cells from non-regulatory lymphocytes after interaction with DC. Plasmacytoid DC seem to possess high potential, as they express significant levels of the recently-identified glucocorticoid-induced TNFR family-related receptor (GITR)-ligand that can drive Treg cell expansion [102, 103]. Interestingly, while these investigations relate to tolerance induction, recent publications remind us of the potent role of DC as initiators of immune responses. A potential new function has been attributed to them: the control of Treg cell activity. In vitro studies have shown that DC matured with TLR ligands (e.g. LPS or CpG) can stimulate naïve T cells and render them refractory to Treg cell suppression, at least in terms of proliferation [104, 105]. Although these preliminary observations require more formal/rigorous testing, a potential role for this activity of DC cannot be excluded, including in the transplant settings. Thus every DC therapy aimed at generating new

Treg cells must take into account this potential deleterious (T cell stimulatory) effect.

48.8.3
Fingerprints of "Tolerogenic" Dendritic Cells

Another important anticipated advance is more detailed characterization of the so-called "tolerogenic" DC. Apart from the initial paradigm of immature DC as tolerogenic DC, it has become evident that a scientific definition of the different states in which DC are associated with tolerance induction is missing. Indeed, there are very few reports that have identified distinctive features of tolerogenic DC. Chang et al. [42] have indicated a key role for the inhibitory receptors ILT3 and ILT4, that are highly expressed on the surface of human APC after interaction with $CD8^+CD28^-$ suppressor T cells. Moreover, they have reported the tolerogenic properties of the APC cell line KG1 after its genetic manipulation to increase ILT3 and ILT4 expression [42]. Experiments involving primary DC forced to express those molecules will be informative. Studies by Grohmann et al. [106, 107] have clearly indicated that DC treated with CTLA4-Ig or with ligands for CD200Rs upregulate the expression of IDO (probably through an indirect effect mediated by IFN-γ). Under these conditions, DC exhibit tolerogenic properties, being able to suppress T-cell proliferation and induce T-cell apoptosis by production of toxic metabolites. It will be valuable to analyze the gene expression profile of the various types of tolerogenic DC described: pharmacologically-manipulated DC, DC obtained from tolerant allograft recipients, and tolerogenic DC obtained in vitro after interaction with Treg cells. This approach will allow fundamental comparisons between the various cell population and define potential molecular signatures of the tolerogenic status, that will inevitably become targets for new tolerance-inducing approaches.

48.8.4
Dendritic Cells at the Crossroads of the Immune System

Successful clinical application of DC-based therapies will depend on the ability to extrapolate from small animal model systems to outbred large animal (including non-human primate) studies and then to human organ transplantation. It has been demonstrated that transplant patients are characterized by the presence of circulating memory T cells able to cross-recognize allo-Ags [108, 109]. These T cells have lower requirements (e.g. co-stimulation level) for activation and, furthermore, do not require the presence of lymph nodes to be activated [110]. It will be very important to investigate the capacity of tolerogenic DC to induce tolerance in memory T cells and to determine any additional requirements needed to obtain this result in comparison to naïve T cells. Moreover, there is a consistent genetic polymorphism between individuals that may be reflected in different actions of the immune system to the same type of insult. In addition, transplant recipients are commonly characterized by an abnormal health state that can influence the type of response generated by the host immune system. To cope with these potentially

challenging issues, extensive understanding of the bi-directional interactions that occur between DC and not only T and B cells, but also all other elements of the innate immune response (e.g. NK cells, γδ T cells, NKT cells) [111] will be necessary. We envisage that, with this knowledge, it will become clear which conditions are needed to establish the aforementioned feedback loop between tolerogenic and regulatory T cells that may characterize the state of stable tolerance.

The awareness of potential pitfalls not considered in the early design of DC-based therapies together with appropriate regimen optimization studies, are likely to bring the concept of using DC to promote transplant tolerance into the clinic.

48.9 Conclusions

The advent of immunosuppressive drugs has allowed the pretentious concept of organ transplantation to become reality and a now widely-used service procedure. Unfortunately, the cost of this success is defined by the non-specificity of these drugs, which results in systemic inhibition of the functionality of the immune system. The practical consequence for the transplant recipient is a higher incidence of infections and an increased predisposition to tumor development. Moreover, with the exception of very rare cases, the immunosuppressive therapy does not allow/instruct the recipient's immune system to become tolerant to the transplanted tissue and consequently, lifelong immunosuppressive therapy is required (with manifestation of the toxicity of the drugs due to their long-term use). Knowledge gained of the central role of DC as controllers of immune reactivity has now indicated that a new therapeutic approach based on their tolerogenic function may help achieve the long-sought goal of drug-free transplant tolerance. DC with tolerogenic function can be obtained using a variety of manipulations. These cells can be employed to instruct the recipient's immune system to tolerate donor alloAg with a selective effect that does not alter immune reactivity to other Ags. Animal models have clearly indicated the feasibility and the advantages of this new approach, but they have also reminded us of the remarkable plasticity of DC functions (that justifies interest in these cells for therapy of such diverse clinical conditions as cancer, autoimmune disease, and graft rejection). Considerable potential resides in the patient's own DC for immune regulation and the time has arrived for design of human-oriented protocols that can be tested in the clinic.

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XVII Allergy, Asthma

49 Nickel Presentation to T Cells in Contact Hypersensitivity

H. U. Weltzien, K. Gamerdinger and H.-J. Thierse

49.1 Introduction

The two types of allergy causing the majority of allergy-related health hazards in the human population are the IgE-mediated type I allergy, mostly affecting respiratory and mucosal tissue, and the skin-affecting contact hypersensitivity (CHS), classified as type IV allergy [1]. This contribution focuses on CHS, which presents itself as a clearly T-cell mediated disease. Contact allergens, upon skin contact and penetration, are taken up and processed by skin-residing Langerhans cells (LC) representing the phenotype of immature DC. During this so-called sensitization phase, contact sensitizers typically induce an activation and maturation process in LC [2, 3], resulting amongst others in the expression of chemokine receptors and migration to the draining lymph node. It is there where the then matured DC activates naïve, MHC-restricted αβ T cells, which equipped with appropriate homing receptors return to the skin [4]. Secondary skin-contact with the same allergen in what is called the elicitation phase then activates these memory-type T cells to produce inflammatory cytokines, which finally results in the typical skin lesions.

In most cases skin-sensitizers are represented by low molecular chemicals or drugs (haptens). Such haptens [5], when bound to proteins, may induce hapten-specific immune responses on the level of B cells as well as of T cells. The fact that T cells specifically react to haptens in an MHC-restricted manner has been known since 1974 [6]. However, it was only in the 1990s that hapten epitopes for T cells were identified as covalently modified peptides, bound to the peptide binding grooves of class I or class II MHC molecules [7–14]. Such epitopes may be created either by intracellular processing of hapten-modified proteins [15] or by modification of already MHC-associated peptides, albeit with low efficacy [16]. In contrast,

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haptens bound directly to amino acid side chains of the MHC proteins themselves have as yet not been found to be recognized by T cells [17]. In this sense haptens appear as quite "normal" antigens for T cells, mimicking a foreign amino acid side chain of a peptide associated with self-MHC. The relevance of hapten-peptide determinants in sensitization for CHS has been demonstrated by *in vivo* immunization with class II or class I MHC-binding TNP-peptides [12, 18].

The picture gets slightly more complicated by the observation that in industrialized countries more contact allergies are caused by nickel-containing metal alloys than by any other chemical or drug, with more than 10% of the female population sensitized [19, 20]. Unlike "classical" haptens, which usually attach irreversibly to MHC-associated peptides via one covalent bond, metal ions including Ni^{2+} engage up to six ligands in a reversible coordination complex [21]. This reversibility of binding and the multiplicity of contacts imposed significant difficulties on the characterization of Ni-epitopes and raised questions of whether or not the presentation of metal ions to T cells by DC and other APC resembled the presentation of classical haptens [22].

49.2
Molecular Basis of Nickel Presentation to Human T Cells

49.2.1
CD4-positive T Cells

Despite observations which stress the effector role of CD8-positive T cells in CHS in general [18, 23] and in Ni contact dermatitis in particular [24], the majority of information available on Ni recognition by T cells relies on $CD4^{+}$ T cell clones. $CD4^{+}$ T cells with specificity for Ni^{2+} ions may be activated and cloned from peripheral blood or skin lesions of Ni-allergic individuals and have been a target of research for more than 20 years [25–29]. However, Ni^{2+}-reactive CD4 T cells can also be isolated from blood of non-allergic individuals [30], particularly after removal of $CD8^{+}$ cells [31]. *In vivo*, skin-residing LC were soon assigned a crucial role in the presentation of Ni^{2+} to T cells [32]. In studies aiming at the identification of the structural basis of Ni-MHC epitopes it was noticed that Ni-reactive T cells varied greatly in terms of their cross-reactivity to other metals as well as in their requirement for actively processing APC [27, 28]. Stimulation with live or aldehyde-fixed APC divided Ni-reactive T cell clones about equally into those recognizing Ni^{2+} exclusively on metabolically active APC, versus those independent of APC metabolism, i.e. of antigen processing [33]. Both of these groups contained clones reactive to Ni-pulsed APC as well as clones requiring for activation the permanent presence of Ni-salts in the medium [33]. Pulsing of APC in this context stands for removal of unbound Ni^{2+} after incubation of APC with $NiSO_4$. Moreover, despite the fact that most Ni-reactive T cells appeared as classically MHC-restricted clones [28, 34], others have been found to exhibit extremely promiscuous HLA-restriction [35].

49.2.1.1 Presentation of Hhapten-like Ni Epitopes

A first indirect indication that Ni^{2+} might be recognized by T cells similar to other haptens in association with MHC-bound peptides came from an experiment of Romagnoli et al. in 1991 [36]. These authors demonstrated that the proliferation of a T cell clone specific for a malaria peptide was inhibited by $NiSO_4$, and that this inhibition depended on the presence of a histidine in the peptide's sequence. However, direct proof that Ni^{2+} on MHC-bound peptides could be recognized by Ni^{2+}-specific T cells turned out to be extremely difficult.

Further indirect evidence came from observations that a notable fraction of *in vitro* activated, HLA-restricted, Ni^{2+}-reactive T cell clones proliferated in response to Ni-pulsed APC [28]. Most importantly, T cell clones and TCR transfectants were described which recognized Ni^{2+} in the context of their restricting HLA alleles on some, but not on all types of APC [37, 38].

Peripheral blood of individuals exhibiting exceptionally strong Ni-sensitization was found to contain an unusually high proportion of T cells expressing the Vβ17 element in the variable portion of their TCR β-chains [39, 40]. Moreover, some of these $V\beta17^+$ β-chains were found to be extremely similar in sequence and exchangeable between homologous TCR without loss of specificity [41]. However, specificity for Ni^{2+} was not defined by Vβ17 in a superantigen-like manner, but was determined by N-region encoded amino acids within the 3rd hypervariable β-chain region in combination with selective α-chains [41].

More recently, Lu et al. performed a detailed investigation of the epitope recognized by one of these $V\beta17^+$ clones, designated ANi2.3 [42]. Clone ANi2.3 was restricted to the HLA-DR13 associated DR52c allele. However, only DR52c expressed on professional APC such as B cells was capable of presenting Ni^{2+} to ANi2.3. Other DR52c expressing cells could be transformed into Ni-presenters by addition of MHC-peptide extracts from DR52c expressing B cells. Thus, a restricted proportion of DR52c bound peptides appeared to be involved in Ni presentation.

On the other hand, a histidine in position 81 of the DR52c β-chain, situated in close proximity to the peptide binding groove, proved indispensable for activation of ANi2.3 by Ni^{2+} [42]. Histidine as a classical coordination partner for Ni^{2+} implied that here, unlike in the case of covalently binding haptens, direct interactions of the metal ion with MHC amino acids had to be considered. Taking into account that Ni^{2+} activation of clone ANi2.3 was independent of antigen processing by APC and effective even with Ni^{2+} pulsed, fixed APC, these data clearly indicated a hapten-like ligand formed by coordination of Ni^{2+} with His81 of the MHC and one or more amino acids of an MHC-associated peptide [42]. This situation is depicted schematically in Fig. 49.1.

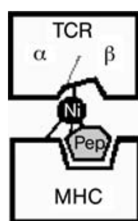

Fig. 49.1 Hapten-like presentation of Ni^{2+}. Ni^{2+} ions attach to multiple coordination sites provided by amino acid side chains of MHC-associated peptides as well as of the MHC protein [42]. The alteration is recognized by TCR like a peptide modification by classical haptens.

49.2.1.2 **Nickel Ions as TCR-MHC Crosslinkers**

The identification of the Ni^{2+}/peptide/MHC ligand for clone ANi2.3 mentioned above appeared to confirm the original hypothesis of Romagnoli et al. [36], envisaging the determinants for Ni-specific T cells as coordination complexes of Ni^{2+} with MHC-bound peptides. However, as pointed out in the introduction, many Ni-reactive T cells require activation conditions significantly differing from those for clone ANi2.3. Thus, another HLA-DR restricted, $V\beta 17^+$ clone (SE9) from a different donor, unlike ANi2.3 could not be activated by Ni^{2+}-pulsed APC, but required the constant presence of free $NiSO_4$ in the medium [35]. On the other hand it proliferated in response to Ni^{2+} in the presence of aldehyde-fixed APC, indicating independence of antigen processing. In this case, Ni^{2+} recognition was independent of the nature of HLA-bound peptides, and His81 on the HLA-DR β-chain, despite being indispensable for SE9 activation, was not sufficient to immobilize Ni^{2+} on the DR surface [43]. The additional identification of two tyrosine residues in hypervariable regions of the TCR α-chain as possible Ni^{2+} contacts resulted in the assumption that a perfect Ni^{2+} coordination site for clone SE9 was provided only by intermediate formation of TCR-MHC associates, which subsequently were stabilized via the Ni^{2+} coordination complex [43, 44]. This type of TCR activation by Ni^{2+} is very different, indeed, from the classical view of MHC-restricted antigenic epitopes: the binding of the antigenic entity requires for TCR signaling the pre-formation of a TCR-MHC complex, which in itself is not stable enough to activate the signaling cascade. This view is based on the observation that positively selected peripheral T cells require for survival repeated contacts of their TCR with the restricting MHC structures [45, 46]. A schematic representation of the complexation of Ni^{2+} with coordination sites provided by coordination sites of TCR and MHC, independent of its associated peptide is depicted in Fig. 49.2.

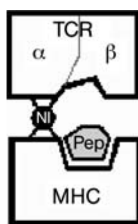

Fig. 49.2 Ni^{2+} as a crosslinker for TCR and MHC. Coordination sites on TCR and MHC are distributed in a way to result in effective binding of Ni^{2+} only upon pre-association of the two proteins. This binding may or may not involve amino acids of the MHC-associated peptides [43].

49.2.1.3 **Cryptic Peptide Epitopes**

The data reviewed so far resulted from only two individual Ni^{2+}-reactive, HLA-restricted, $CD4^+$ T cell clones. This implies that many different ways of Ni-presentation for T cells will have to be considered. Amongst other possibilities, one has to envisage that ions of Ni and possibly other transition metals may affect peptide-binding to MHC molecules, or that they may interfere with the protein processing machinery of DC and other APC. The latter possibility has been demonstrated for gold-specific T cells. Some supposedly gold-reactive TCR were found to react to MHC-associated cryptic peptides, which resulted from altered protein processing

as a result of metal-induced oxidative processes [47]. In fact, such reactivities of "metal-specific" T cells to absolutely metal-free cryptic peptides may partially explain some surprising T cell cross-reactivities for different metal ions [48].

49.2.2 CD8-positive T Cells and Non-HLA Restricted Nickel Presentation

$CD8^+$ cytotoxic T cells are major players in the pathology of contact dermatitis [18, 23, 49–51]. In fact, *in vitro* activation of T cells from peripheral blood of Ni-allergic donors with $NiSO_4$ results in substantial proliferation of $CD8^+$ cells [33]. Moreover, Cavani et al. produced evidence that Ni-induced proliferation of $CD8^+$ T cells represents a crucial difference between allergic and non-allergic individuals [24, 52]. However, cloning and particularly the long-term cultivation of $CD8^+$ T cells with reactivity for Ni^{2+} implicates significantly more experimental difficulties than the perpetuation of $CD4^+$ human T cells. The rare studies on $CD8^+$, Ni-reactive clones reveal that most of them, like the $CD4^+$ ones, are clearly HLA-restricted [33]. However, some $CD8^+$, cytotoxic clones were isolated from different donors, which were activated by Ni^{2+} independent of HLA class I or class II molecules [33, 53]. These clones required the presentation of Ni^{2+} on human cells (murine APC were ineffective), and their αβTCR were the only specificity-determining membrane structures. The restricting human membrane elements have not yet been identified, but nonclassical HLA molecules such as Cd1 or MIC-A and -B could be excluded [53]. Since the clones also did not classify as NKT cells, these data indicate a probably very different way of Ni-presentation to such T cells. On the other hand, these clones, except for the lack of HLA restriction, rather resembled typical cytotoxic T cells, including the mechanism of perforin-mediated cytolysis [53].

49.3 Nickel-Binding Proteins

49.3.1 A Role for Carrier Proteins in Nickel Presentation

Ni^{2+} ions, upon entry into the outermost layers of the skin, are not expected to remain as free soluble entities. Due to their potential to form coordination complexes with electrophilic ligands they will, with great probability, quickly find themselves attached to cell surfaces or soluble proteins. Binding to cells has been reviewed in Section 49.2.1.1, revealing T-cell reactivity to Ni-pulsed APC. The best studied Ni-binding soluble protein in humans is serum albumin (HSA) whose N-terminal amino acids Asp-Ala-His-Lys provide a perfect square planar coordination site for Ni^{2+} and Cu^{2+} [54-56]. While previously major emphasis in this context was put on the detoxifying properties of HSA [57], a carrier potential of HSA, revealing Ni^{2+} transfer to other peptides or proteins, also became apparent [58]. Since HSA is particularly abundant in skin [54], studies have been initiated to determine the

role of HSA as a potential Ni^{2+} carrier in nickel contact dermatitis [59]. These investigations remarkably demonstrated that on a molar basis HSA-Ni complexes stimulated Ni-reactive human T cell clones as effectively as $NiSO_4$ or $NiCl_2$. Thus, in this situation, HSA did not remove Ni^{2+} from the system, but rather functioned as a store from which Ni^{2+} could be transferred to TCR/MHC-defined coordination sites. In contrast, nickel-toxicity towards mammalian cells as determined by apoptosis was reduced by the addition of HSA (HJT, unpublished results).

For a number of reasons it appeared that HSA itself did not provide the peptide-part of the antigenic determinant. Thus, HSA rather appeared as a transporter or carrier molecule for Ni^{2+}, or/and as a mediator of activation [59]. An interesting observation in these studies was that unlike $NiSO_4$, HSA-Ni was incapable of pulsing APC for recognition by clone ANi2.3. This suggests that the HLA-DR/peptide-defined Ni-binding site on DR52c-positive APC (see Section 49.2.1.1) exhibits too low affinity for Ni^{2+} to liberate the metal ions from their coordination site on HSA. Such transfer apparently needs the pre-formation of a short-lived, as such non-activating TCR-MHC association. If this complex, as in the case of the clones ANi2.3 or SE9, supplies a Ni-coordination site of equal or higher affinity than the one in HSA, the metal ions may be transferred and the complex stabilized by cross-linking [59].

Fig. 49.3 shows a schematic representation of this situation. It implies that carrier molecules such as HSA may on the one hand detoxify Ni^{2+} by preventing its uncontrolled interaction with cellular proteins. On the other hand, they facilitate the specific transfer of Ni^{2+} to high-affinity cellular acceptor sites.

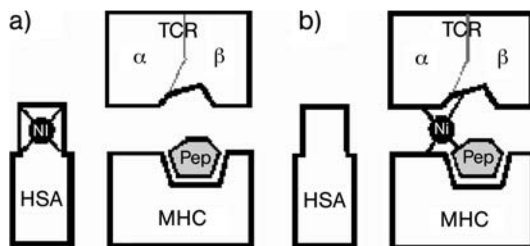

Fig. 49.3 Transfer of Ni^{2+} from carrier proteins to TCR/MHC-defined coordination sites. Carrier proteins (HSA or others) remove and detoxify free nickel ions from body fluids by complexation. Transfer of metal ions to other proteins is limited to those providing coordination sites of comparable or higher affinity for Ni^{2+}. The majority of TCR/MHC combinations will, therefore, not detach Ni^{2+} from its carrier (a). However, if suitable coordination sites are supplied by short-lived complexes between TCR and self-MHC, Ni^{2+} may cross-link and stabilize these complexes (b), eventually resulting in TCR activation and signaling [59].

49.3.2 Heatshock Proteins as Nickel Binders

Our *in vitro* studies addressing the possible involvement of HSA in transport and transfer of Ni^{2+} to contact sites between T cells and APC do not tell us whether *in*

vivo HSA, indeed, plays a central part as a Ni-carrier. Other proteins in dermis or epidermis not yet recognized as Ni-binding entities may be involved as well or may even be superior to HSA. In this context it is important to realize that proteins with particularly high affinity for Ni^{2+} are, in fact, less likely to deliver the metal ions to TCR-MHC interfaces than Ni-binders with lower affinity. Too low affinity, on the other hand, will also not be desirable. Optimal Ni-carriers will be represented by proteins exhibiting Ni-affinity high enough to allow for complexation over reasonable intervals of time, and low enough to permit transfer of Ni^{2+} to high affinity coordination sites.

Another unresolved issue is the question whether such Ni-carriers transfer Ni^{2+} directly to TCR-MHC interfaces, or whether Ni^{2+} is first delivered to intermediate contact sites on or within the cells involved in immunological responses. Since no physiological role for Ni^{2+} is known in mammals, very little information is available on Ni-binding proteins in humans. We have, therefore, recently initiated investigations to identify Ni-binding proteins in human T cells, B cells, DC and keratinocytes by 2-dimensional electrophoresis combined with mass spectroscopy. Even though these studies are still in an early phase, they already gained numerous interesting results. Thus, immobilized affinity enrichment of proteins from B cell lysates on Ni-NTA beads resulted in at least 150 protein spots, visible upon silver staining. Due to lower sensitivity this number was substantially reduced on Coomassie stained gels, but 28 spots have already been identified [60]. Quite unexpectedly, 16 of these 28 proteins belonged to the group of stress-inducible heat-shock proteins (HSPs) or chaperonins, including HSP-60, HSP-70 and the oligomeric complex of TriC/CCT. The latter one was actually among those proteins most impressively enriched by immobilized Ni^{2+}. Thus Ni^{2+}, in addition to the formation of MHC-associated epitopes recognizable by the acquired immune system, intimately interacts with essential constituents of the innate defense system. It will be interesting to determine whether HSPs serve as transfer molecules between soluble protein carriers such as HSA and the TCR/MHC interphase or/and as mediators for so-called danger signals [61] to alert DC and the general immune responsiveness.

49.4 Concluding Remarks

Although nickel is a major sensitizer for contact dermatitis, it is by far not the only metal that causes T cell-mediated adverse immune reactions. Thus, cross-sensitization of Ni-allergic patients to Pd, Cu or Co is rather common [27, 28] and also CHS to Cr, Au and Be is well documented [48, 62–65]. Moreover, metal-specific T cells are obligatory in chronic beryllium disease (CBD) [66] as well as in cobalt-induced hard metal lung disease (HMD) [67]. We therefore tend to envisage the studies on Ni presentation reviewed here as a general model for the presentation of metal ions to T cells.

Most importantly, these studies indicate that the term "presentation" may apply only in part to Ni^{2+} and other metal ions. Only a minority of antigenic epitopes, like the one for clone ANi2.3 [42], may be formed *in vitro* by stable MHC-association of Ni^{2+}. But even in this case the number of coordination sites on the MHC-peptide surface appear not to be sufficient to compete for Ni^{2+} bound to intermediate carrier molecules such as HSA. *In vivo* we expect the vast majority of Ni^{2+} ions to be carrier-associated, which makes the formation of stable MHC/peptide/Ni^{2+} epitopes extremely unlikely. We, therefore, propose in accordance with our model in Fig. 49.3 that (a) Ni^{2+} can only cross-link the αβTCR of T cells with MHC/peptide surfaces on DC or other APC if these complexes provide a high affinity coordination site of at least four ligands, and (b) that this requires an as such unstable association of the TCR in question with the MHC allele that initiated its positive selection in the thymus. The model gets complicated by the widely accepted view that in CHS Langerhans cells first have to migrate to the local lymph node, mature to DC, and only then and there present the allergen to T cells exposing an appropriate TCR. This implies that the Langerhans cell needs to carry Ni^{2+} from the skin to the lymph node, bound to an acceptor site, which by all probability is not the MHC protein itself. Here come into play cellular Ni-binders such as proteins recently identified by us [22, 60]. Of particular interest in this context are Ni-binding heat-shock proteins, some of which are known to facilitate the peptide loading of MHC molecules [68, 69].

Another finding of probably broad impact is the involvement of the conserved His81 in HLA-DR β-chains as a potential coordination site for Ni^{2+} in two independent T cell clones. The fact that His81 is conserved in all DR alleles except for DR53 goes along with our finding that a majority of human Ni-reactive CD4 T cells is restricted to HLA-DR [39]. This also may explain the high frequency of Ni allergy as well as the fact that no particular HLA-association has been observed in nickel contact dermatitis [70]. It is interesting to note that a comparable, though different situation has been described for Be^{2+}-specific T cells isolated from CBD patients. In this case a remarkable association of CBD with the HLA-DP2 allele was correlated to glutamic acid in position 69 of the DP2 β-chain as one essential coordination site for Be^{2+} [71, 72].

In conclusion, in our view the molecular basis of nickel presentation by DC or other APC differs significantly from that of antigenic peptides or classical haptens. It appears that as a rule metal ions, in contrast to peptides or haptens, do not form stable MHC-associated epitopes on the presenting cell, at least under *in vivo* conditions. Instead, they require short-lived, non-stimulating complexes of TCR with self-MHC, which they effectively may crosslink if the TCR/MHC-interphase provides suitable coordination geometry. These coordination sites may or may not involve amino acids of the MHC-bound peptides, they may involve one or both chains of the αβTCR, but they clearly require a high degree of specificity in arranging suitable amino acid side chains into a geometrically exactly fitting alignment. Hence, in terms of specificity nickel-reactivity is much closer to classical peptide recognition than to superantigen reactivity.

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50
Dendritic Cells in Asthma

Hamida Hammad and Bart N. Lambrecht

50.1
Introduction

Allergic asthma is one of the most common chronic diseases in Western society, characterized by variable airway obstruction, mucus hypersecretion and infiltration of the airway wall with Th2 cells, eosinophils, and mast cells. If we are to devise new causal therapies for this disease, it is important to elucidate how Th2 cells are activated and respond to intrinsically harmless allergens. Dendritic cells (DCs) are the most important antigen presenting cells (APC) in the lung and are mainly recognized for their exceptional potential to generate a primary immune response and sensitization to aeroallergens. Much less attention has been paid to the role of DCs in established inflammation. Based on functional studies in a murine model for asthma, we propose that DCs are essential for generating allergen specific effector Th2 responses in ongoing inflammation in sensitized mice.

50.2
Asthma as a Th2 Driven Disorder

Asthma is a chronic inflammatory disorder of the airways, characterized by the clinicopathologic symptoms of intermittent and reversible airway obstruction, enhanced mucus production, chronic eosinophilic inflammation and bronchial smooth muscle cell hypertrophy, eventually leading to impaired epithelial repair and airway remodeling. It is generally believed that asthma is controlled by Th2 cells secreting IL-4, IL-5, IL-9, IL-10, IL-13 and GM-CSF, whereas mast cells and eosinophils exert important effector functions. Under physiological conditions, activated Th2 cells induce an immune response to fight extracellular or parasitic infections. In allergic asthma, IL-4 and IL-13 induce the expression of cell adhesion molecules on inflamed endothelium and epithelial production of chemokines leading to the recruitment of inflammatory cells, stimulate the production of IgE by B cells and cause bronchial hyperreactivity. IL-5 is important for the growth, differen-

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tiation and activation of tissue eosinophils. IL-9 is important for mast cell growth and activation. Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the growth of eosinophils and the activation of APC [1, 2] (see Fig. 50.1). Moreover, cytokines produced by Th2 cells and inflammatory cells can affect the airway epithelium, subepithelial (myo)-fibroblasts, and smooth muscle cells in the lungs thus leading to structural abnormalities. As effector Th2 lymphocytes are intimately involved in controlling the various aspects of this disease, elucidating the mechanisms by which these cells are generated in response to allergen encounter (i.e. allergic sensitization) and subsequently activated to cause disease upon antigen challenge is crucial for development of a preventive and/or curative strategy.

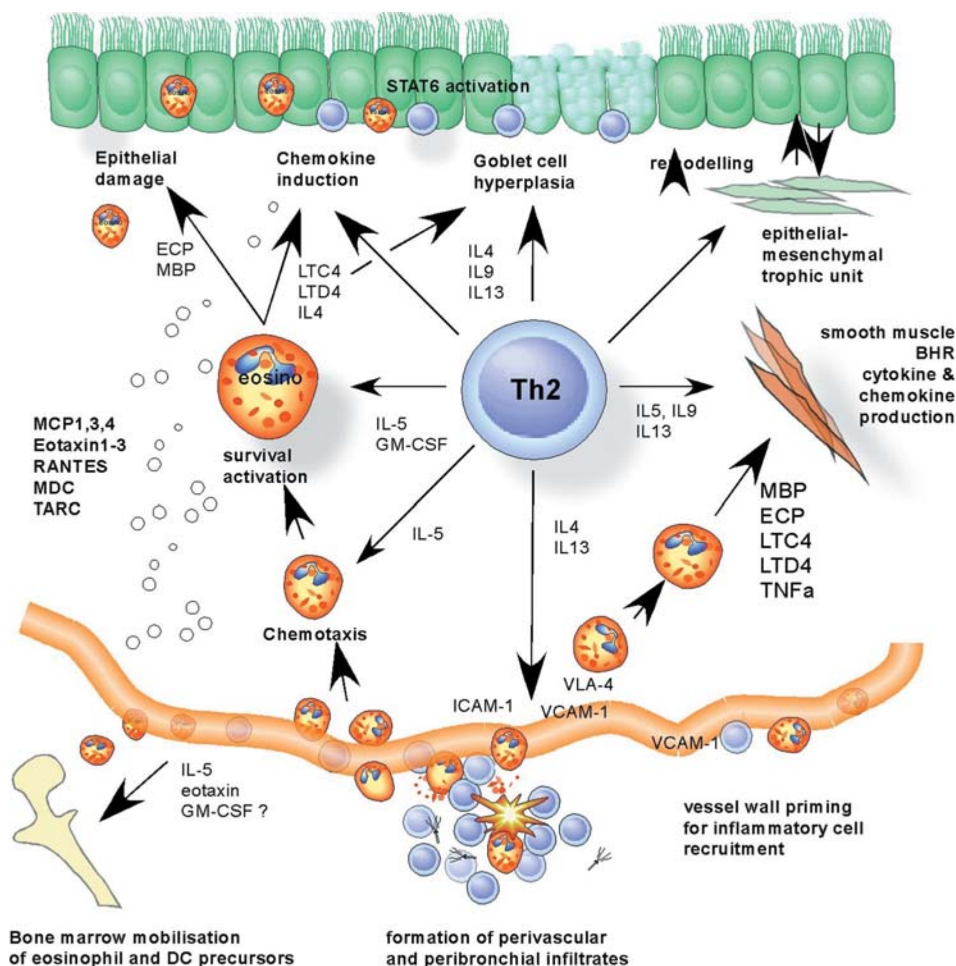

Fig. 50.1 Effector functions of Th2 cells. Effector Th2 cells produce several cytokines and chemokines which exert their effects on eosinophils, airway smooth muscle, epithelium and endothelium, resulting in several pathologic characteristics of asthma.

50.3 Lung Dendritic Cell Subsets

Dendritic cells are distributed throughout the whole lung and can be found in every compartment including the conducting airways, lung parenchyma, alveolar space, visceral pleura, and the pulmonary vascular bed [3–8].

In the conducting airways, $CD11c^{high}$ DCs form a dense network underneath and within the epithelium with dendrite projections towards the lumen to sample for environmental antigens. In mice and rats, these cells are mainly of the myeloid origin and show a high turnover of about 2–3 days under steady state conditions [7, 9]. In mice, they express CD11c, MHC II, and CD11b, and low to intermediate levels of DEC205 (unpublished observations). These DCs do not express CD8, a marker present on a subset of lymphoid organ DCs, or the pDC marker Gr-1 [10]. Very few studies have addressed the presence of DCs in human conducting airways. Irregularly shaped cells with a marker pattern comparable to that of blood-derived DC (positive for DR, DQ, L25, RFD1, CD1c, and CD68) were predominantly observed in the epithelium and subepithelial tissue of the bronch(iol)us and in the bronchus-associated lymphoid tissue. In the epithelium, only approximately 30% of these cells were positive for CD1a (OKT6) and demonstrated Birbeck granules typical of Langerhans cells. In the subepithelial tissue, DC formed characteristic small clusters with T cells [5, 11, 12]. The precise origin of conducting airway DCs is unknown. In humans, DCs in the mucosa express CCR6 [13], a receptor for the MIP-3α a chemokine produced abundantly by bronchial epithelial cells [14].

Some studies have also looked at the phenotype and function of interstitial lung DCs obtained after enzymatically digesting a piece of a lung lobe. In humans, rats and mice, interstitial DCs are again predominantly $CD11c^{+}$ myeloid and are immature as assessed by the low expression of the co-stimulatory molecules CD40, CD80, CD86 [10, 15–18] and by the high expression of several receptors for inflammatory chemokines and endocytic receptors [19]. It is generally believed that interstitial lung DCs have a much longer half life of about 10 days [7].

In recent years a $CD11c^{dim}$ murine plasmacytoid DC population has been characterized and is found in all lymphoid tissue and bone marrow. These cells are the main source of both IFN-α and IFN-β when stimulated with bacterial CpG or by viral infection. The early progenitors of pDCs have been described within the bone marrow hematopoietic cells, and express Ly6-C and CD31 [20, 21]. This pDC population has been ignored for a long time because of its expression of the B cell marker B220 and of the granulocyte marker Gr-1 [22]. Plasmacytoid DCs differ from myeloid DCs by their expression of high levels of CD45RA, 120G8, and 440c, and their low expression of CD11c and MHC class II [23, 24]. For a long time, it was thought that pDCs were absent from the lung as several groups have failed to detect them [12]. The finding that pDC were virtually absent from the lower conducting airways contrasted with previous findings in upper airway allergy [25]. Recently however, one group has been able to identify a population of pDCs in enzymatic lung digests of humans [26]. Like their counterparts in the blood, lung pDCs are characterized by the expression of CD123 and BDCA-2. However, the anatom-

ical location of human pDCs in the lung has not yet been characterized. In mouse lung, pDCs were predominantly found in lung interstitium and expressed low levels of CD11c, and were positive for Gr-1, B220 and 120G8 as previously described in lymphoid tissues [27], and produced large amounts of IFNα in response to influenza virus infection. In baseline noninflammatory conditions, these cells are very immature expressing very low levels of co-stimulatory molecules, and intermediate levels of PDL-1 [16].

One easily accessible lung compartment that contains DCs is the alveolar compartment. In humans, alveolar DCs are low autofluorescent cells expressing CD11c and HLA-DR, and are very immature as indicated by their low expression of co-stimulatory molecules and CD83 [11]. In rats, the phenotype of alveolar DCs more closely resembles the phenotype of intra-epithelial DCs, in being OX62 positive [28]. More recent data in the mouse suggest a particularly critical role for alveolar DCs in retaining antigens for a prolonged time. These DCs are positive for F4/80, CD11c and CD11b, and capture airborne antigens and maintain the capacity to activate specific T cells long after antigen exposure [29]. In steady state, these cells comprise a minor fraction of alveolar cells, but they expand considerably in the lungs with ongoing Th2 immune responses [29]. In mice, alveolar DCs also express the transcription factor, RunX3 conferring susceptibility to TGFβ regulation [30].

50.4
Function of Lung Dendritic Cells in Primary Immune Responses to Inhaled Antigen and Sensitization to Inhaled Allergen

Dendritic cells are crucial in regulating the immune response by bridging innate and adaptive immunity. Signals from the type of antigen and the response of the innate immune system to it are translated by DCs into a signal that can be read by the cells of the adaptive immune response leading to an optimal response for a particular insult. Together, these signals consist of provision of a particular density of peptide-MHC, the expression of co-stimulatory or Th polarizing cell surface molecules and the expression of soluble cytokines and chemokines that polarize T cells or enhance their survival. At the same time, DCs also control the function and expansion of regulatory T (Treg) cells that tightly control overzealous inflammatory T-cell responses. Although controversial, it has been suggested over recent years that particular functions of DCs such as tolerance or immunity or Th1/Th2 differentiation might be a specialized function of defined subtypes of DCs [31, 32]. Others have refuted this idea and have claimed that DCs are very versatile cells, and can virtually induce any type of response depending on the need of the moment [33].

Immature DCs are distributed throughout the lung and are at the focal control point determining the induction of pulmonary immunity or tolerance [34–36]. Airway DCs form a dense network in the lung ideally placed to sample inhaled antigens and these cells migrate to draining lymph nodes (LNs) to stimulate naïve T cells [9, 37, 38]. Just as in the gut, airway DCs extend long dendrites to the lumen

of the airways, forming bud-like extensions at the border of the air interface [39]. Within a few hours after inhalation, airway myeloid DCs and plasmacytoid DCs have taken up fluorescently labeled antigen within the draining mediastinal LNs [16, 38, 40]. After 24 h, both mDCs and pDCs in the mediastinal LNs contain antigen inside vesicles of the cytoplasm. What is unclear at present is whether pDCs take up antigen in the periphery of the lung and subsequently migrate to the nodes, or whether antigen is being transported to them by migratory mDCs or even a specific subset of CD8α^- CD11b$^-$ migratory DCs recently described by Belz et al. [41]. Transport of immunogenic material from one nonmigratory DC to another is certainly a possibility, as CD8α^+ DCs injected into the lung induce an immune response in the mediastinal node without migrating into it [42]. Under steady state conditions, mDCs continuously migrate to draining LNs and present either (self)-auto antigens or harmless antigen in a tolerogenic form (see Fig. 50.2) [43]. Once they have reached the draining LNs, mDCs express intermediate levels of co-stimulatory molecules and MHC II. Under most conditions, DC migration is linked to partial activation, and even in the absence of infection, the majority of epithelial derived DCs arrive in the mediastinal nodes in a partially mature CD86$^+$ CD40$^+$ state [44].

Recent studies have suggested that myeloid lung DCs mediate protective immunity to inhaled antigens only when properly activated by innate immune system activating immune signals, acting through Toll-like receptors or other pattern recognition receptors (Fig. 50.2). Under inflammatory conditions such as those provided by LPS or virus infection, the expansion of T cells induced by myeloid DCs leads to the generation of Th1 or Th2 effector cells in the mediastinal nodes [45, 46]. The signals that determine the type of response after encountering a pathogen in the lung are delivered by DCs in the lymph node. Reis e Sousa et al, recently suggested that DC maturation and provision of peptide-MHC to T cells is not sufficient to generate effector cells [47]. Cytokines are dominant signals that determine the quality and quantity of an effector immune response. During generation of an efficient effector immune response, DCs also have to overcome suppression by Treg cells, and the dominant way by which they seem to do this is by producing IL-6, that releases the suppression by naturally occurring Tregs [48]. Certain pathogens or pathogen-derived products induce the direct secretion of Th1 polarizing cytokines by DCs. Alternatively, NK cells reacting to pathogens can be an important source of initial IFN-γ for developing a strong Th1 response. Although it was recently shown that DCs in skin-draining lymph nodes recruit and activate NK cells as a source of IFN-γ, this pathway has not yet been demonstrated in the lung, a normally Th2 biased compartment [49]. Stumbles et al. and Dodge et al. showed that resting respiratory tract DCs mainly induced Th2 responses [18, 50]. As a direct proof that myeloid DCs can induce Th2 sensitization to inhaled allergen, it was shown that intratracheal injection of bone marrow derived mDCs pulsed with OVA induced a Th2 response to OVA and subsequently led to severe features of asthma when mice were rechallenged with OVA aerosol [51].

The type of immune response induced by mDCs also depends on the strength of the activating innate immune system stimulus. Elegant studies by Eisenbarth

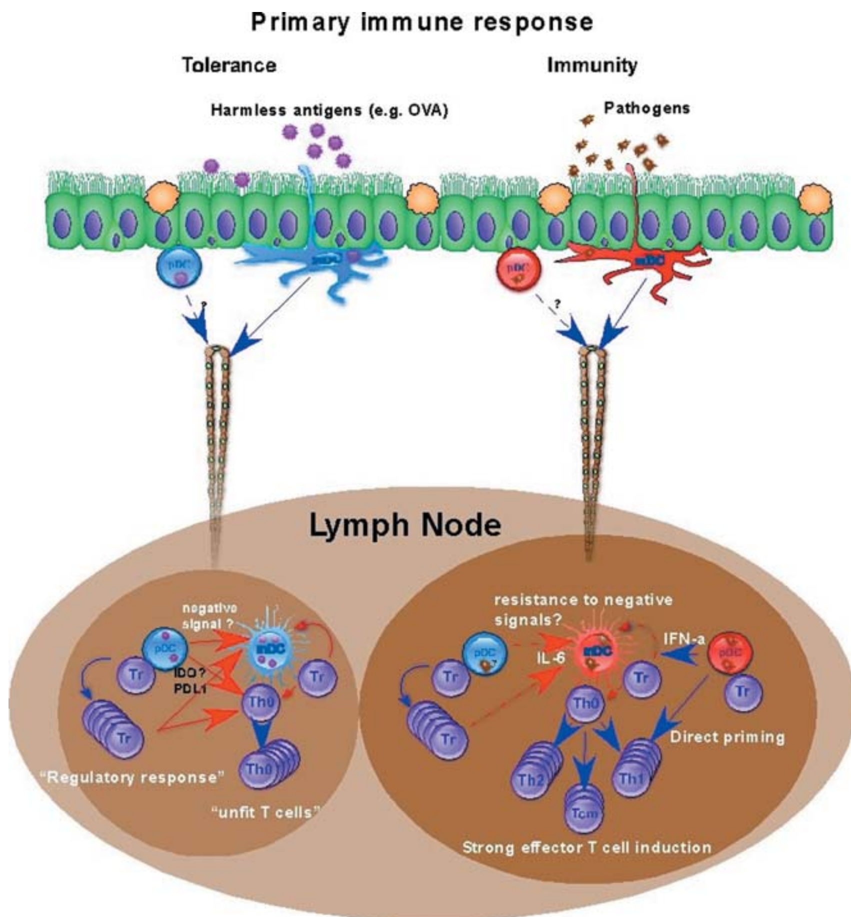

Fig. 50.2 Immune regulation by mDCs and pDCs in the lung. Under steady-state conditions (left), in the absence of accompanying danger signals in the lung, inhaled antigens are picked up by mDCs and pDCs, which take the antigen to the mediastinal nodes. Here, partially mature mDCs induce a short lived boost of division in antigen specific T cells, but these T cells fail to differentiate into effector cells and die. Some T cells might also differentiate into Treg cells. Plasmacytoid DCs in the draining node influence the generation of T effector cells from dividing T cells probably by giving negative signals (IDO, PDL-1) to T cells and mDCs. At the same time they alo generate Treg cells. Under inflammatory conditions (right), mDCs now arrive in the draining nodes as fully mature cells, resitant to Treg suppression. Ag specific T cells again undergo proliferation, but this time generating effector cells. On the other hand, pDCs also acquire a mature phenotype and prime Ag specific T cells to become effector cells as well.

showed that low level TLR4 agonists prime mDCs to induce a Th2 response, by inducing their full maturation, yet not their production of IL-12 [46]. High level LPS administration induced high level IL-12. These findings might help to explain the effects of environmental exposure to LPS on the reduced incidence of allergic sensitization. IL-12 seems to be a dominant cytokine for Th1 responses in the lung, yet the LPS induced Th1 response induced by myeloid DCs in the lung was not dependent on IL-12 [52]. Although interleukin 12 may be redundant for some Th1-inducing stimuli, it is certainly sufficient as retroviral over-expression of this cytokine in myeloid DCs in the lung induced strongly polarized Th1 responses [53].

50.5
Is Tolerance Induction in the Lung a Property of Specialized Dendritic Cell Subsets?

Even if DCs can induce priming to inhaled antigen, still the predominant outcome of inhalation of harmless antigens is tolerance. This is shown best for the model allergen ovalbumin (OVA). When given to the airways of naïve mice via aerosolization, nasal droplet aspiration or intratracheal injection, it renders mice tolerant to a subsequent immunization with OVA in adjuvant, and effectively inhibits the development of airway inflammation, a feature of true immunological tolerance [16, 34, 54]. Although DCs carrying harmless OVA antigen induce a vigorous proliferation in naïve antigen-specific T cells, the outcome of the response is tolerance and not immunity [16, 45, 55]. One possible explanation is that the (self) antigens encountered by airway DCs under noninflammatory conditions cannot induce a full activation of DCs sufficient to induce T-cell effector function, and therefore do not serve as "effector DCs" [47]. Partially mature DCs would then induce an abortive immune response, leading to the generation either of regulatory T cells [56–58] or of unfit T cells, that fail to reach the threshold for survival and are finally deleted (see Fig. 50.2) [59].

A recent study showed that mediastinal $CD8\alpha^-$ mDCs of OVA-tolerized mice produce high levels of IL-10 and induce the generation of a protective population of Th2-like regulatory T cells expressing both Fox p3 and GATA-3 [34]. This response required the presence of surface ICOS-L and CD86 [35, 60]. Another paper from the same group demonstrated that the transfer of splenic $CD8\alpha^+$ DCs but not of $CD8\alpha^-$ DCs of mice immunized with OVA and heat-killed *Listeria* could protect against asthma development through the induction of Th1-like regulatory cells [61]. These regulatory cells produced both IL-10 and IFN-γ, express T-bet and Fox p 3, indicating that the Th1-like regulatory cell population generated is different from Tr1 cells [62] or from *in vitro*-generated regulatory T cells [63]. Although the $CD8\alpha^+$ DC subset has not yet been described in the lungs, these findings suggest that a spectrum of adaptive regulatory T-cell types can be generated in the lung depending on the type of antigen encountered and the subset of DCs involved. Some bacteria have taken advantage of this DC-induced regulatory T-cell induction to their own advantage. Mycobacteria or *Bordetella pertussis* bind to DC-SIGN on the surface of myeloid DCs, leading to a decrease in the expression of CD80, CD83,

and CD86. At the same time, lung DCs increase their production of IL-10 and promote the generation of regulatory T cells [64, 65], leading to a dampening of efficient effector T-cell responses in the lung.

We recently found that pDCs in the lung are able to take up harmless antigen, migrate to draining lymph node to induce tolerance through the induction of regulatory T cells [16]. Strikingly, the depletion of pDCs with either depleting anti-Gr-1 antibodies or with 120G8 led to a break of inhalational tolerance to this harmless antigen and induced a very strong Th2-dependent pulmonary inflammatory reaction typical of asthma, showing that pDCs provide intrinsic protection against Th2 sensitization in the lung. We also recently showed that the adoptive transfer of OVA-pulsed pDCs could completely suppress the development of cardinal features of asthma [16], likely through the generation of regulatory T cells. A possible explanation for the tolerogenic potential of pDCs might be that pDCs can produce indoleamine 2,3-dioxygenase (IDO), which has a strong inhibitory activity on T-cell proliferation [66], and inhibits inflammatory airway disease (see Fig. 50.2) [67]. Interestingly, IDO expression has been demonstrated recently in pulmonary CD11c$^+$ cells although the exact cell type involved has not clearly been identified [68]. Another explanation for the tolerogenic properties of pDCs is related to their immature phenotype, as it has been demonstrated that immature DCs can induce regulatory T cells [56–58]. In contrast to immature pDCs, activated pDCs can augment cell surface expression of MHC class II and co-stimulatory molecules, increasing their T-cell stimulatory ability and become immunogenic. It is tempting to speculate that this conversion of pDCs of tolerogenic to immunogenic cells could be an explanation why infections with respiratory viruses lead to a break in inhalational tolerance and are often associated with enhanced allergic responses to harmless antigens (see Fig. 50.2) [69–71].

50.6
Accumulation of Mature Dendritic Cells in Ongoing Asthmatic Inflammation

Despite these known functions of DCs on primary immune responses and allergic sensitization, it is less clear if airway DCs are also necessary for the presentation of allergen to resting memory Th2 and/or effector Th2 cells during a secondary immune response. In favor of DCs, it is known that the number and maturation state of lung DCs is elevated during secondary immune challenge with allergens and during chronic airway inflammation [12, 72, 73]. This suggests that DCs are functionally involved in presenting allergens to T cells and thus control airway inflammation [9, 15, 36, 74]. Arguing against a role for DCs, is the notion that previously primed T cells have less need for co-stimulation in comparison to naïve T cells, which led to the hypothesis that any peripheral APC expressing MHCII could stimulate effector Th2 cells [75–77]. Some intriguing findings in an animal model of asthma support a predominant role for DCs in presenting antigen during secondary immune responses as they occur in chronic asthma. Upon allergen challenge to the lung, CD11c$^+$ cells are strongly recruited to the lung and acquire a mature

phenotype expressing essential co-stimulatory molecules (CD80, CD40, ICAM-1, PDL-1, PDL-2) when resident within the inflamed lung [73, 74, 78]. Adoptive transfer experiments demonstrated that accumulation of airway DCs was not due to reduced migration to the mediastinal lymph nodes. Rather, the massive increase in airway DCs was supported by an almost 3-fold expansion of myeloid CD31^{hi} Ly-6-C^{neg} hematopoietic precursor cells in the bone marrow. When these BM precursors were grown in GM-CSF they differentiated into MHCII^{+} CD11c^{+} DC, but when grown in IL-5 they generated eosinophils, illustrating their pluripotent character. As these cells express the CCR3 eotaxin receptor, a serum rise in eotaxin might be the signal that increases the output of DCs and eosinophils at times when they are needed in the airways [28, 73].

In all these studies, CD11c^{+} DCs accumulate within sites of eosinophilic airway inflammation and cluster with CD4^{+} T cells in the airway submucosa and the perivascular space at areas of intense eosinophilic inflammation, forming multiple contacts with CD4^{+} T cells (see Fig. 50.3) [17, 78]. The consequences of this interaction with T cells have been studied elegantly by Huh et al. in a rat model of asthma. After a single OVA aerosol challenge in OVA-sensitized rats, airway DCs accumulated in the airway mucosa and submucosa and formed contacts with primed T cells in turn inducing the up regulation of co-stimulatory molecules on otherwise immature airway DCs [15]. These authors hypothesized that contact with primed T cells turns immature mucosal DCs from an antigen uptake to an antigen-presenting mode leading to local antigen presentation to mucosal T cells. The accumulation of DCs in the airways is so strong that the bone marrow increases its output of myeloid progenitors for DCs and also for eosinophils [28, 73].

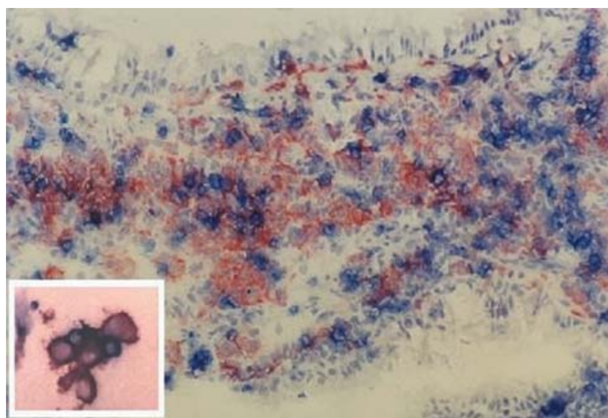

Fig. 50.3 Staining of CD4^{+} T cells (membrane bound blue) and CD11c^{+} DCs (red) in the lung of OVA sensitized and challenged mice visualizes co-localization of CD4^{+} T cells and CD11c^{+} DCs within peribronchial sites of inflammation. Goblet cell hyperplasia is seen.

50.7 Direct Proof for a Functional Role for Dendritic Cells in Stimulating Effector Th2 Responses

As intense local interactions between primed CD4+ T cells and locally matured DCs are a feature of eosinophilic airway inflammation across species, we studied whether effector CD4+ T cells functionally depend on DCs to exert their effector functions and in this way orchestrate airway inflammation [17]. In human studies it is almost impossible to study the functional role of DCs because it is not possible to deplete DCs and study the subsequent inflammatory response to inhaled allergen. We addressed this question by using a novel system of conditional depletion of CD11c+ cells expressing a DT receptor transgene under the control of the CD11c promoter [17, 79]. Systemic administration of DT to these mice leads to the rapid reduction in splenic and lymph node CD11c+ DCs, and concomitantly to an absence of the primary cytotoxic T-cell responses to *Listeria* infection or experimental administration of protein antigens. We adopted this strategy and administered the DT locally via i.t. injection, leading to a loss of CD11c+ cells in the BAL fluid and lung tissues (DCs and macrophages) and draining MLN (DCs) [17]. Using this unique system, we conditionally depleted CD11c+ cells locally from the lung immediately prior to OVA challenge in OVA-sensitized mice and saw that the cardinal features of asthma, such as eosinophilic airway inflammation, goblet cell hyperplasia and bronchial hyperreactivity to metacholine, failed to develop. Similarly, asthmatic features were also absent when DCs were depleted from already fully inflamed airways. As these features critically depend on CD4+ T cells and their secreted products, the most likely explanation was that effector function was not induced in the absence of CD11c+ cells. In support of this theory, we saw that in the absence of CD11c+ cells, *ex vivo*-isolated CD4+ cells produced greatly diminished amounts of IL-4, IL-5 and IL-13. We then adoptively transferred *in vitro*-primed CD4+ Th2 cells from DO11.10 TCR transgenic T cells in CD11c+-depleted mice. Previous data have shown that such *in vitro*-differentiated Th2 cells mimic *in vivo*-generated memory T cells and can be used to transfer passive Th2 reactivity to OVA aerosol [80]. However, when Th2 cells were adoptively transferred in mice that were depleted of CD11c+ cells, no Th2 effector cytokines IL-4, IL-5 and IL-13 were produced by the T cells in the draining lymph nodes of the lung, whereas they readily did in the presence of lung CD11c+ DCs. All the salient features of asthma were restored by adoptive transfer of wild type bone marrow-derived CD11c+ myeloid DCs, but not by transfer of CD11c+ wild type alveolar macrophages. These data of local depletion of CD11c+ DCs immediately prior to antigen challenge to the lung, support our previous work in which we systemically depleted all myeloid DCs from the lungs, lymph nodes, bone marrow and spleen of HIV-LTR promoter driven thymidine kinase transgenic mice prior to allergen challenge [9]. These data indicate that dendritic cells are essential for the development and maintenance of secondary immune responses, but are they also sufficient? Allergen sensitized mice were challenged with only DCs in the absence of any adjuvant or allergen aerosol. These mice developed a similar eosinophilic airway inflammation,

Th2 cytokine synthesis and bronchial hyperreactivity as seen after a regularly used aerosol challenge [17, 78].

50.8 Determinants of Dendritic Cell Driven Th2 Responses in Asthma

Based on studies on the functional interaction between mucosal T cells and DCs [15] and based upon the data from mice in which DCs were depleted from challenged lungs, it is clear that effector Th2 responses *in vivo* in the lung continuously depend on antigen presenting DCs. One possible explanation would be that effector T cells *in vivo* remain dependent on co-stimulation in contrast to their *in vitro* counterparts [75, 81]. Numerous models of asthma have demonstrated that blocking the interaction of co-stimulatory molecules of the B7 superfamily (CD80, CD86, ICOS-L) or TNF-R family (OX40L) can reduce the features of asthma, even when given during challenge in sensitized mice and even when given together with *in vitro* primed Th2 cells [82–86]. As lung DCs are the predominant cell type expressing CD80/CD86 family members at times of allergen challenge, an absence of DCs might have the same effect as blocking these co-stimulatory molecules [15, 74, 78]. However, challenge with DCs derived from bone marrow of CD80/86 double knockout mice in sensitized mice induced similarly strong airway inflammation as seen after challenge with wild type DCs. It is likely that other co-stimulatory molecules besides CD80/CD86 are involved in activating T cells in secondary immune responses. Several molecules on the surface of the DC, like B7RP-1 (also known as ICOSL), OX-40L, 4-1BBL, CD40, and ICAM-1 have been reported to have co-stimulatory capacity and can be responsible for the induced reaction in the absence of CD80/CD86 [87]; a likely candidate in this context is B7RP-1 (ICOSL), induced on mature DCs in lung draining lymph nodes [35]. Blockade of ICOS-ICOS-L using an ICOS-Ig fusion protein was able to completely suppress asthmatic features induced by adoptive transfer of OVA-specific Th2 cells to the airways of mice [82]. However, blocking ICOS-ICOSL interaction with an anti-ICOS antibody during CD80/86$^{-/-}$ DC challenge in sensitized mice did not reduce significantly airway eosinophilia, although there was a trend towards reduced airway eosinophilia [78]. One explanation for the poor efficacy of ICOS in this model in contrast to previous models would be the recent observation that DCs downregulate the expression of B7RP-1 during eosinophilic airway inflammation [74, 78]. Strikingly, we also observed an increase in the B7 family members PDL-1 and PDL-2, ligands of the inhibitory PD-1 receptor, on DCs within eosinophilic inflammation [78]. Although signaling through PD-1 is generally seen as an inhibitory signal, recent data suggest that PDL-1 might also provide a co-stimulatory signal to T cells [88]. In support, blocking PDL-1/PDL-2 using PD1-Fc has the potential to suppress eosinophilic airway inflammation (B.L. and H. Kuipers, unpublished work). Another co-stimulatory pathway able to compensate for the lack of CD80/86/B7RP-1 co-stimulation on DCs, would be OX-40L. OX-40 (CD134), a member of the TNFR family, is a major regulator of anti-apoptotic proteins such as Bcl-xL and Bcl-2 and strong-

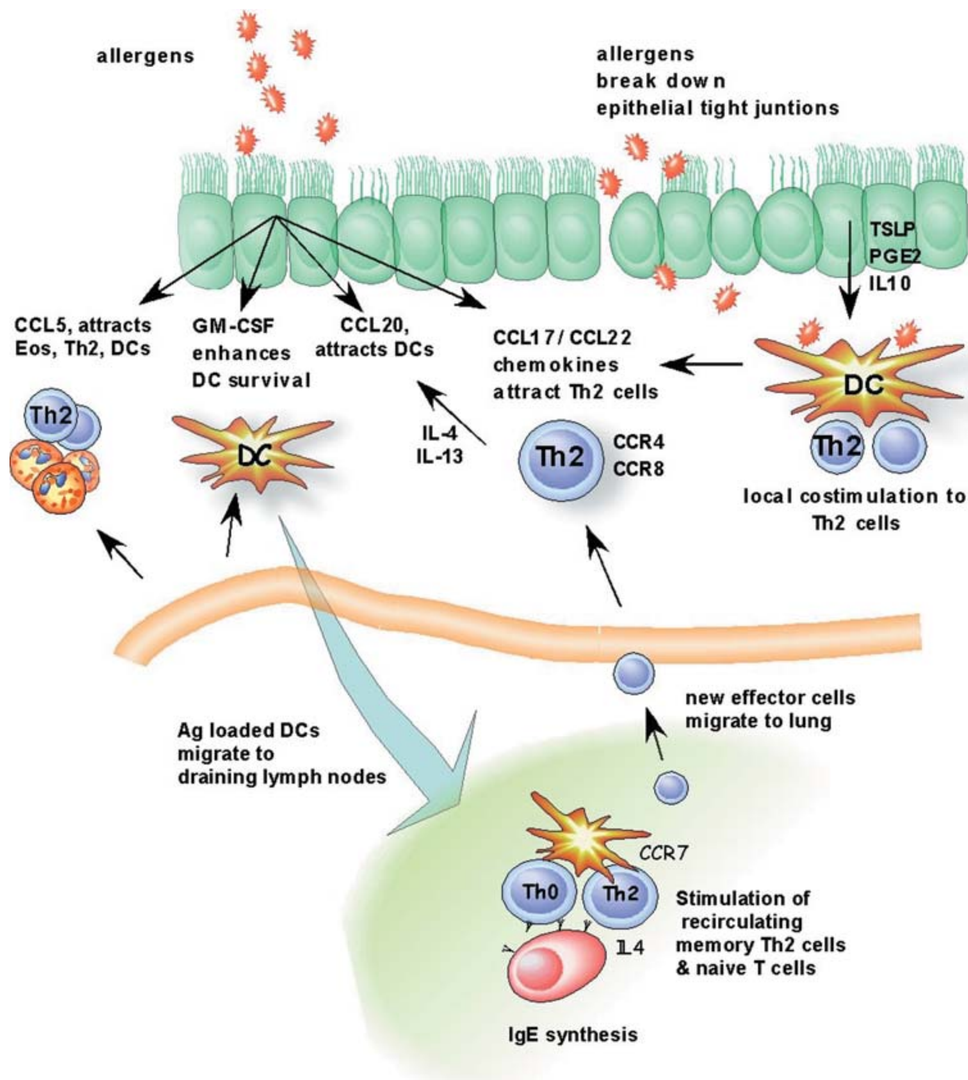

Fig. 50.4 Interaction between epithelial cells and dendritic cells during established inflammation. Allergens stimulate epithelial cells to release chemokines and growth factors for DCs, Th2 cells and eosinophils. The recruited DCs are also stimulated by allergen and produce even more chemokines for Th2 cells. Locally attracted Th2 cells interact with DCs in the airways, leading to local DC maturation and T-cell co-stimulation of effector cytokine production. These activated Th2 cells eventually control the inflammatory process by activating eosinophils and mast cells and by feeding back on the epithelium and DCs.cell.

ly promotes the survival of antigen-activated primary CD4 T cells. In addition, OX-40 is preferentially expressed by memory Th2 cells. Blocking OX40-OX-40L interaction impaired all features of asthma induced by adoptive transfer of OVA-specific Th2 cells [89].

An alternative explanation would be that lung DCs are essential for the recruitment of Th2 cells by producing Th2 selective chemokines. It was indeed shown that lung $CD11c^+$ DCs within sites of eosinophilic airway inflammation and human monocyte derived DCs exposed to the house dust mite allergen Der p 1 produce high amounts of the Th2 selective chemokine TARC (CCL17), acting on $CCR4^+$ Th2 cells (see Fig. 50.4) [74, 90]. Therefore, depletion of lung DCs might lead to an "unfavorable" chemokine gradient, failing to attract primed Th2 cells to the lung.

50.9 Dendritic Cells in Human Asthma

Although it has not been proven directly in humans that DCs are responsible for the Th2 sensitization process, some *in vitro* findings strongly imply these cells. The way in which allergens are handled by DCs is fundamentally different between atopics and non-atopic individuals [91–94]. When DCs obtained from house dust mite (HDM)-sensitive asthmatics were exposed to the endotoxin-free major allergen component Der p1 *in vitro*, they mainly produced IL-10, but little IL-12. They expressed the co-stimulatory molecules CD86 and PDL1 and secreted the TH2-cell-specific chemokines CCL22 and CCL17 [90, 95]. When monocyte-derived DCs from non-HDM-allergic donors or non-allergic donors were exposed to Der p1 they mainly produced IL-12, expressed CD80 and produced the TH1-cellspecific chemokine CXCL10. Not surprisingly, monocyte-derived DCs from allergic patients induced TH2-cell responses of naïve alloreactive T cells *in vitro*, whereas those DCs from non-allergic individuals induced Th1 responses. Therefore, the way HDM is handled by DCs is crucial to the generation of Th2-cell sensitization, and is clearly different in patients with allergy to HDM. The cysteine protease activity of Der p1 induced these changes in the DCs of allergic individuals, indicating that the activation of a protease-activated receptor on DCs leads to aberrant cellular activation in patients with asthma [95]. The enzyme activity of Der p1 could also indirectly facilitate antigen presentation by DCs *in vivo*, by allowing access to intra-epithelial DCs through cleavage of epithelial tight junctions and by locally activating the release of epithelial GM-CSF [96, 97]. In this way, the epithelial response to allergens might also determine the type of adaptive immune response induced by DCs. Supporting this idea, DCs treated with lipase (an industrial allergen displaying an enzymatic activity) have been reported to induce a strong recall CD4+ T-cell response associated with a high production of IL-4 and IL-13, and a low production of IFN-γ [98]. However, allergens without enzymatic activity can also directly activate DCs to induce Th2 priming. For instance, phytoprostane lipids contained in pollen allergens

can induce DC maturation and inhibit IL-12 production by LPS-activated DCs. When co-cultured with allogeneic naïve T cells, pollen-treated DCs polarized the immune response towards Th2 [99].

In clinical asthma, allergen challenge leads to an accumulation of myeloid, but not plasmacytoid DCs to the airways of asthmatics, concomitantly with a reduction in circulating CD11c+ cells, showing that these cells are recruited from the bloodstream in response to allergen challenge [12, 100]. In stable asthma, the number of CD1a+ DCs is increased in the airway epithelium and lamina propria, and these numbers are reduced by treatment with inhaled corticosteroids [72]. Based on the above argumentation in mice studies of asthma, it is very likely that part of the efficacy of inhaled steroids might be due to their effects in dampening airway DC function. According to current thinking, epithelial dysfunction, either intrinsic to asthma or caused by persistent inflammation, leads to epithelial release of pro-fibrotic cytokines such as epidermal growth factor and transforming growth factor-β acting on fibroblasts and smooth muscle cells, disturbing the equilibrium between epithelial destruction and growth and repair. Moreover, asthmatic epithelium might release factors such as GM-CSF, TSLP or chemokines that profoundly influence DC survival and/or function (Fig. 50.4). The exact consequences of this epithelial remodeling on the functioning of the airway DCs are currently unknown. Finally, many inflammatory cell types such as mast cells, basophils and eosinophils are recruited to the airways in chronic asthma. These cells release many mediators such as cytokines, neuropeptides, enzymes, and lipid mediators that may also profoundly influence DC function and in this way might perpetuate ongoing inflammation [36]. As only one example, it is known that histamine and PGD2, both released by mast cells upon cross linking, reduce the potential of DCs to produce bioactive IL-12, and in this way contribute to Th2 polarization [40, 101].

The exact role of plasmacytoid DCs in ongoing allergen specific responses in asthma is currently unknown. It was shown that pDCs accumulate in the nose, but not lungs, of allergen challenged atopics [25]. When pDCs were pulsed with pollen allergens, they were as efficient as mDCs in inducing Th2 proliferation and effector function [102]. Others have suggested, as in the mouse, that pDCs might also confer protection agains allergic responses. In children at high risk of developing atopic disease, the number of circulating pDCs was reduced [103].

50.10 Conclusion

Airway dendritic cells are crucial in determining the functional outcome of allergen inhalation in the lung and antigen presentation by myeloid DCs leads to Th2 sensitization typical of allergic disease. It is increasingly clear that DCs have an antigen presenting function beyond sensitization. DCs therefore constitute a novel target for the development of anti-asthma therapy aimed at the origin of the inflammatory cascade.

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XVIII
Cancer

51
Dendritic Cells in Human Cancer

Casey A. Carlos and Olivera J. Finn

51.1
Introduction

Since Coley's observations in the early part of the 20th century, that an immune response to a fulminant bacterial infection can also eliminate an established tumor [1], medicine has been interested in the ability of the immune system to combat cancer. Cells of the innate and the adaptive immune system are capable of not only protecting an organism from external threats, but also controlling the wellness of the body internally by maintaining normal body homeostasis. Tumors are a double challenge to the immune system. They are both a "foreign" threat presented by the transformed phenotype of the tumor cells, as well as a "self" that has lost normal homeostasis. Although there is a lot of evidence for tumor immunity as well as for success of immunotherapy of cancer, there are also many examples where the immune system can contribute to tumor growth. Furthermore, the cancer cells growing in the presence of immune cells can develop ways to circumvent normal immune functions. Dendritic cells, as coordinators of the immune response, are very important in ensuring tumor recognition by the immune system, but they can also be hijacked by the tumor for subversion of the antitumor responses. In this chapter we will discuss the important role for DC in priming effective immune responses to tumors, the changes in tumor cells that can be recognized by DC leading them to alert the immune system to the presence of the tumor, and finally, how the tumor and the tumor micro-environment can divert DC function from what is good for the immune system to what is good for the tumor.

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51.2 Dendritic Cell Functions that are Important for Effective Immunity against Cancer

As discussed in previous chapters, DC are uniquely able to prime naïve T cells [2]. It is known that tumors express a variety of proteins either de novo or in aberrant forms that can be recognized by the adaptive immune system [3]. To achieve immune stimulation DC must take up tumor antigens, migrate to the lymph nodes and present these antigens to T cells in an immunostimulatory context. Tumor-derived proteins can also be brought into the lymph node through the afferent lymphatics, where they can be taken up by lymph node resident DC. DC take up antigens through receptor-mediated endocytosis or receptor independent pinocytosis. These antigens are either soluble products from tumor cells or apoptotic or necrotic tumor cells. As will be discussed below, the source of antigen is critically important. For example, apoptotic and necrotic tumor cells can respectively suppress or enhance DC maturation [4, 5]. Both $CD8^+$ and $CD4^+$ T cells are required for an effective antitumor response [2, 6]. Most of the experiments to date suggest that to achieve effective destruction of tumors, DC priming of naïve T cells should result in the activation of $CD4^+$ helper T cells of the Th1 type and expansion of $CD8^+$ T cells capable of direct tumor cell lysis (CTL) [6]. DC elicit this type of immunity best in their fully mature state when they produce inflammatory cytokines such IL-12 and express high levels of co-stimulatory molecules.

Tumor antigen-specific T cells are found in many cancer patients, providing evidence that a certain level of priming of naïve T cells does take place during the growth of the tumor [7]. However, since the frequency of such cells is usually low and the cancer progresses in spite of their presence, the whole process of T-cell priming is considered to be sub-optimal in cancer. A whole new field of research has developed around understanding the reasons for that and how the immune outcome may be improved by ensuring an optimal DC function. These studies have lead to the development of approaches that use *in vitro* manipulated DC as components of vaccines for cancer (see Chapter 52).

DC are also important in the activation of innate immunity. This activation occurs at the tumor site and can be enhanced by DC maturation or activation in response to endogenous danger signals released by the tumor, molecules such as high mobility group box 1 protein (HMGB-1) or uric acid, which will be discussed in more detail below [8–10]. Most notably, DC have been shown to activate NK cells that are central to antitumor responses. With many tumors downregulating MHC class I molecule expression as a means of escape from adaptive immunity, it is important to be able to activate NK cells that do not require class I molecules to exert antitumor effects [11]. IL-12 produced by DC activates both NK and NKT cells to proliferate, produce IFN-γ, and become cytotoxic effector cells [12, 13]. DC can deliver indirectly the "help" message to T cells by inducing NK cell cytokine production [14, 15]. DC can furthermore induce and control migration into the tumor of other cells of the innate immune system by producing chemokines such as CXCL8, CCL4, CCL17, and CCL22 [16].

51.3 Dendritic Cell Recognition of Malignant Changes in Tissues

The immune system must discriminate between many different stimuli and determine whether to respond by activation of effector cells or establish a state of tolerance and/or anergy. DC appear to play a pivotal role in these decisions by being able to assess through a variety of cell surface receptors different stimuli from the tissue environment, and to process other contextual information such as the molecular characteristics and relative concentration of the stimuli [2, 17]. Multiple changes in tumor cell protein profiles occur during carcinogenesis. How these altered proteins and other molecules affect DC and the subsequent tumor-specific response is a new and growing area of investigation.

There are several examples of proteins released from tumor cells that are recognized by DC. Stress proteins such as heat shock proteins are released during periods of high cell turnover, as seen during tumor growth. It is known, for example, that DC express a receptor CD91 for the heat shock protein gp96 that is released by necrotic cells [18–20]. By binding to CD91 and activating DC, gp96 sends a danger signal to the immune system. Similarly, HMGB1 and uric acid are released by necrotic cells and represent internal danger signals. The receptors for HMGB1 on the DC are Toll-like receptor 2 (TLR2) and RAGE (receptor for advanced glycated end-products) [8, 21]. HMGB-1 function is associated with DNA "bending" and when released from necrotic cells it induces maturation of DC [8]. Uric acid, which exists in the extracellular environment in the form of monosodium urate, is also perceived as a danger signal by DC that transmit that message to the immune system [22].

Proteins can also be differentially post-translationally modified in tumor cells compared to normal cells and this difference can alert the DC. For example, due to changes in the levels of various glycotransferases, tumor cells can add fewer and/or different sugars to glycoproteins or over-sialylate the sugars that have been successfully added. Lower levels of glycosylation expose peptide epitopes that on normal molecules are hidden by large carbohydrate side chains. Tumor-specific carbohydrate moieties, such as the Thomsen–Friedenreich antigen (TF, β-Gal-[1–3]-α-GalNAc-O-serine), also known as the T antigen, and the Tn antigen (α-GalNAc-O-serine/threonine), are recognized by antibodies as tumor markers. Furthermore, these tumor-specific carbohydrates can bind to and crosslink receptors on the surface of immune cells, especially DC and other cells of the innate immune system.

Recognition by DC of new carbohydrate structures on transformed cells can serve as a danger signal to the immune system. One example is recognition by DC of the MUC1 mucin that is over-expressed on tumor cells and aberrantly glycosylated with tumor-specific sugars such as T and Tn described above. It has been shown that tumor forms of MUC1 bind to receptors on the surface of cells of the innate immune system, such as the mannose receptor, sialoadhesin (Siglec-1), and Gal/GalNAc-specific calcium dependent lectins [23–25]. Tumor MUC1 can alter

the differentiation of monocytes into DC *in vitro* [26]. Monocytes co-cultured with MUC1$^+$ tumors or their supernatants in the presence of GM-CSF and IL-4 become DC that express low levels of co-stimulatory molecules and increased IL-10 production. These DC do not make high levels of IL-12 and induce greater number of IL-4 producing CD4$^+$ T cells [26]. It has been shown that MUC1, through its exposed peptide epitopes in the VNTR (variable number of tandem repeats) region can induce chemotaxis of immature human myeloid DC [27]. Furthermore, the carbohydrate side chains on the tumor form of MUC1, which have a high concentration of sialic acid residues, can increase expression of co-stimulatory molecules, such as CD40, CD80 and CD86, as well as CD40 and MHC-Class II on human myeloid DC, comparable to the effects seen after interaction of DC with LPS. The result of the interaction of tumor MUC1 with DC, however, does not result in the induction of IL-12 seen after interaction with LPS, but instead these DC produce IL-6 and TNF-α. T cells stimulated by such DC produce lower levels of IL-2 and higher levels of IL-5 and IL-13 [90].

Gangliosides are another family of molecules aberrantly expressed by cancer cells. Gangliosides are sialic acid-containing glycosphingolipids that are components of the plasma membrane. Gangliosides produced by tumor cells differ from those on normal cells. For example, GM3 is the only ganglioside made by normal melanocytes but melanoma cells produce a wide variety of gangliosides [28, 29]. These tumor gangliosides have been shown to bind to siglec receptors [30] and impair DC development and function [31]. DC matured in the presence of the ganglioside G(D1a) showed decreased levels of co-stimulatory molecules CD40 and CD80 and impaired production of inflammatory cytokines IL-6, IL-12 and TNF-α [32]. These DC were also poor stimulators of T-cell proliferation [32].

51.4 How Tumors Interfere with Normal Dendritic Cell Function

51.4.1 Inhibition of Maturation and Differentiation

There are a variety of mechanisms underlying tumor escape from immune recognition and destruction [33] and many of these involve subverting DC function. Cancer is associated with chronic inflammation [4, 34–36], especially in its formative stages. Once the tumor is established, patients have fewer circulating DC [37–40]. The loss of DC seems to be more pronounced in the myeloid than in the plasmacytoid DC compartment [39, 40]. Removal of the tumor can sometime help restore the number of circulating myeloid DC [37, 39, 40]. DC can be found in rapidly growing tumors and they usually do not express co-stimulatory molecules CD80 and CD86 and have reduced T-cell stimulatory activity [41–44]. Tumors can block or induce abnormalities in DC development by 1) blocking the development of mature and functional DC; 2) increasing the number of immature DC that cannot upregulate MHC class II, co-stimulatory molecules, and inflammatory cyto-

kine production; and 3) inducing production Gr-1$^+$ immature myeloid cells (myeloid suppressor cells) [33, 45]. Tumors make TGF-β, IL-10, PGE_2, VEGF and other molecules that exert their effects on DC and other immune cells [46]. For example, PGE_2 through its receptor EP2 inhibits DC differentiation and function [47, 48].

The recent observation of an abundant population of cells in cancer patients, termed immature myeloid cells (ImC), has helped explain in part the low number and impaired function of DC in some cancer patients. These cells are also found in mouse models of tumors [33, 49]. Tumors alter hematopoiesis by the production of factors that induce ImC [50, 51]. ImC express Gr-1, CD11b, and low levels of MHC class II and low to no co-stimulatory molecules [52, 53]. The presence of ImC is bad for antitumor immunity not only because it represents the failed development of DC but, in addition, these cells are capable of suppressing IFN-γ production by CD8$^+$ T cells in an antigen specific manner [37].

Another newly discovered reason for insufficient DC in cancer patients is the VEGF-induced recruitment of immature DC to become endothelial cells [54]. This is also of interest for basic DC biology because DC have been considered terminally differentiated cells, which is not supported by this observation. This, along with another recent observation that immature DC can become osteoclasts [55], suggests that under proper, or in cancer improper influences, immature DC can transdifferentiate into other cell types.

The production of VEGF is another way that cancer cells prevent maturation of DC. VEGF causes dysfunctional maturation of DC (*in vitro* and *in vivo*) through impaired NFκB activation [56–58], which can be corrected with antibodies against VEGF [59]. Furthermore, activation of Stat3 in tumor cells is implicated in upregulation of IL-10 and VEGF, and impaired maturation of DC resulting in T-cell tolerance [60]. Tumors also make NO, PGE_2, IL-10, and IFN-α, IL-12 p40 homodimers and TGF-β, all of which can lead to the generation of tolerogenic or regulatory T cells [61, 62]. Lastly, tumors can induce expression on DC of molecules that are negative regulators of co-stimulation, such as B7-H1 [63] that selectively induces IL-10 production in resting T cells. It can also induce apoptosis of activated T cells [64].

51.4.2
Influence on Migration

Because the compositions and number of DC at the tumor site is often different from that in normal tissue, it is clear that tumors also alter the ability of DC to migrate to and from the tumor environment. Overall, there is an increase in immature DCs within the tumor mass as opposed to mature DCs that are rarely found within the tumor. Instead mature DCs are found in the peri-tumoral area [65]. There are a number of chemokines produced by tumor cells that can attract immature DC, such as CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL20 and CXCL12. Many of these chemokines are not specific for DC and can attract a variety of immune cells to sites of inflammation and antigen [66].

MUC1 tumor antigen, most unexpectedly, has been found to have a chemokine-like function, being specifically chemoattractive for immature DC implicating it in the selective recruitment of immature DC to $MUC1^+$ tumors [90]. Some tumors are able to selectively attract certain DC subsets. This is clear in the case of ovarian cancer, where there is an increase in plasmacytoid DC at the tumor site. This is thought to occur as a result of the production of CXCL12 (SDF-1) chemokine acting on the CXCR4 receptor on plasmacytoid DC [67]. Plasmacytoid DC, in the absence of strong activation through type I interferons or toll receptor ligands, promote development of regulatory $CD8^+$ T cells [68]. These $CD8^+$ T cells display poor secondary proliferation and cytolytic function, due in large part to the production of IL-10. They also inhibit activation of bystander naïve $CD8^+$ T cells [68].

51.4.3
Suppression of Function

Another recently documented mechanism by which DC from cancer patients induce weak T-cell activation is altered tryptophan catabolism through IDO (indoleamine 2,3-dioxygenase) production. IDO is an enzyme that catabolizes tryptophan in an oxidative reaction. Since tryptophan is an essential amino acid, depletion of tryptophan from the local environment by IDO produced by tumor cells and/or tumor resident DC [69] limits proliferation of immune cells in the tumor area. It has also been postulated that the toxic metabolites of tryptophan catabolism, such as quinolinic acid, kynurenine, and 3-hydroxy-anthranilic acid, may play a role in immunosuppression of T-cell proliferation [70–72]. Interestingly, tumors have hijacked production of IDO as an immune response inhibitor from normal cells involved in immune response regulation. IDO is expressed in subsets of normal myeloid cells, including mouse $CD11c^+CD8a^+$ DC and plasmacytoid DC, and a significant portion of *in vitro* monocyte-derived human DC [73–75]. IDO expression by DC correlates with weak T-cell proliferation in mixed leukocyte reactions, enhanced apoptosis, and weak responses by TCR transgenic $CD8^+$ T cells [74, 76, 77]. This effect can be overcome by the addition of 1-methyl-tryptophan or excess tryptophan *in vitro* [78].

Treatment of DC with IL-10, which is produced by a majority of cancer cells, induces T-cell anergy [79, 80]. T-cell anergy is a state where the T cells become unresponsive to secondary stimulation through the TCR even in the presence of co-stimulatory molecule engagement. Anergic T cells can be generated through the lack of co-stimulation or the presence of regulatory cytokines during primary stimulation [81]. The preponderance of immature DC in cancer would be expected to block T-cell stimulation since they have been shown to induce T-cell anergy *in vitro* and *in vivo* [82–84]. Finally, the tumor-specific T cells interacting with DC at the tumor site may be prevented from forming an effective synapse because of the lack of a proper environment [85]. Whereas in the lymph node, the architecture is supportive of cell–cell interactions, the large number of stromal cells and high concentration of collagen and extracellular matrix molecules at the tumor site can prevent

the close interactions needed between immune cells. In addition to these specific mechanisms identified to date, there is also a frequently observed general down-regulation of the various components of the antigen processing and presentation machinery in DC under the influence of tumor cells [86–89].

Understanding how tumors drive DC dysfunction described above is an important area or research since preventing or overcoming the DC dysfunction has the potential to lead to tumor rejection. The goal is to understand all the mechanisms that the tumor uses to suppress the endogenous immune response and how they might be blocked or otherwise manipulated to improve the DC function. Another goal is to understand what type of an adaptive immune response is possible to elicit by DC that develop and function in the presence of a tumor. These are the DC that are called upon to respond to cancer vaccines administered to patients with cancer. Effective use of therapeutic vaccines will involve measures that can overcome tumor-induced DC dysfunction.

51.5 Summary

Optimal maturation and function of DC are critically important for the development of strong and long-lasting tumor immunity. It is clear from the examples we discussed above, that certain changes that occur during malignant transformation can serve as danger signals picked up by DC and that the DC respond to these changes although not always with a stronger immune response as the final outcome. The weakened overall immune response in cancer patients appears to be mostly a result of a multitude of mechanisms that a tumor develops to divert the DC away from their proper function. Tumors block DC development and suppress DC maturation. Tumors express chemokines that draw immature DC into the suppressive tumor environment. They can even recruit DC to trans-differentiate into endothelial cells as the tumor promotes angiogenesis. They can also block the expression of MHC molecules and co-stimulatory molecules that are important for the induction of a T-cell response. By inducing DC to produces cytokines and molecules that block the Type 1 immune response, cancer cells prevent the few tumor-specific T cells that do get stimulated from being effective against the tumor. With such a large arsenal of DC subverting mechanisms, it is clear that the stimulation of the immune system against cancers, through such strategies as those discussed in Chapter 52, must occur before the tumor overwhelms the body's ability to protect itself.

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Part C
Therapeutical Applications of Dendritic Cells

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XIX
Cancer

52
Dendritic Cell Subsets as Targets and Vectors for Vaccination

Hideki Ueno, Joseph Fay, Jacques Banchereau and A. Karolina Palucka

52.1
Introduction

Early studies in mice have demonstrated that immune system can control tumor growth and metastasis. The identification of T-cell defined tumor antigens in humans facilitated the development of immunotherapy protocols aimed to direct the T cells against tumor. Two independent strategies are being pursued: (1) passive immunotherapy such as adoptive T-cell transfer, and (2) active immunotherapy such as vaccination to induce T-cell immunity *in vivo*. Clinical responses observed after adoptive $CD8^+$ T-cell transfer confirmed the value of tumor-specific T cells in treatment of cancer. Yet, passive immunotherapy is not expected to yield the long-lived tumor-specific T-cell immunity that may be required to prevent tumor progression/relapse. Cancer vaccines aim at inducing both (1) tumor-specific effector T cells, able to reduce/eliminate the tumor mass, and (2) long-lasting tumor-specific memory T cells, able to control tumor relapse. Owing to their capacity to induce and regulate T-cell immunity, dendritic cells (DCs) are increasingly used as adjuvants for vaccination in cancer. Numerous studies in mice showed that tumor antigens loaded DCs are able to induce therapeutic and protective antitumor immunity [1–8] (Table 52.1).

DCs are present in peripheral tissues, where they are poised to capture antigens. These antigens are subsequently processed into small peptides as the DCs mature and move towards the draining secondary lymphoid organs [9]. There, the DCs present the peptides to naïve T cells, thus inducing a cellular immune response, involving both T helper 1 (Th1) type $CD4^+$ T cells and cytolytic $CD8^+$ T cells. Finally, DCs are important in launching humoral immunity through their capacity to activate naïve [10] and memory [11] B cells. DCs can also activate natural killer (NK) cells [12] and NKT cells [13]. Thus, DCs are able to conduct all elements of the im-

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Tab. 52.1 Proof-of-principle studies in mice that formed the basis for DC-based vaccination in humans.

Ex vivo-generated and antigen-loaded DCs induce antigen-specific T-cell immunity *in vivo*
Ex vivo-generated and antigen-loaded DCs can induce humoral immunity *in vivo*
Ex vivo-generated and antigen-loaded DCs induce tumor-specific immunity leading to tumor rejection
Ex vivo-generated DCs are superior to other vaccination strategies in the context of cancer
Ex vivo-generated immature DCs induce tolerance
Combination therapy with *ex vivo*-generated DCs improves vaccine efficacy

mune orchestra and therefore represent a fundamental target and tool for vaccination.

Ex vivo-generated and antigen-loaded DCs have now been used as vaccines to improve immunity [14]. Numerous studies in mice have shown that DCs loaded with tumor antigens can induce therapeutic and protective anti-tumor immunity [15]. The immunogenicity of antigens delivered on DCs has been shown in patients with cancer [14] and chronic HIV infection [16], thus providing a "proof-of-principle" that DC vaccines can work. Yet, the efficacy of therapeutic vaccination in cancer has been questioned [17] because of the undeniably limited rate of objective tumor regressions observed in clinical studies so far. However, the question is not whether DC vaccines work but how to orient further studies to refine the immunological and clinical parameters of DC vaccination to improve their efficacy. We discuss recent progresses in our knowledge of the physiology of DCs – in particular, the identification of distinct DC subsets that induce distinct types of immune response. This, together with an improved understanding of how tumors and the immune system interact will allow us to design better vaccination strategies for patients with cancer.

52.2 Cancer Vaccines

Vaccines against infectious agents represent a success of immunology and have spared countless numbers of people from diseases such as polio, measles, hepatitis B and tetanus [18]. However, progress in vaccination against infectious agents has been largely empirical and not always successful, as many infectious diseases still evade the immune system, most particularly chronic infections such as tuberculosis, malaria and HIV. Further progress will be made through rational design based on our increased understanding of how the immune system works and how the induction of protective immunity is regulated. The same principle applies to cancer vaccines, most particularly as cancer is a chronic disease and by the time it is clinically visible, it has been interacting with and affecting host cells for a long time to ensure its survival.

Vaccination is expected to induce both therapeutic T-cell immunity (tumor-specific effector T cells) and protective T-cell immunity (tumor-specific memory T cells that can control tumor relapse) [19–21]. Numerous approaches for the therapeutic vaccination of humans with cancer have been developed, including: autologous and allogeneic tumor cells (which are often modified to express various cytokines), peptides, proteins and DNA vaccines [14, 21–24]. The observed results are variable, yet in many cases, a tumor-specific immune response has been induced, and tumor regressions, albeit limited, have been produced. These approaches rely on a random encounter of the vaccine with host DCs. A lack of encounter of vaccine antigen with DCs might result in the absence of an immune response. Alternatively, an inappropriate encounter, for example with non-activated DCs or with the "wrong" subset of DCs, may lead to silencing of the immune response [25]. Both of these scenarios may explain some of the shortcomings in current cancer vaccines. Furthermore, we do not know how tumor antigens need to be delivered to DCs *in vivo* in order to elicit an appropriate immune response. Hence the need for studies based on *ex vivo*-generated autologous DCs [26–28] that are loaded with tumor antigen under controlled conditions which might allow us to establish the parameters for optimal vaccination against cancer.

52.3 Dendritic Cells

52.3.1 Dendritic Cell Subsets

Two main DC differentiation pathways are thought to exist (Fig. 52.1) [29, 30]. A myeloid pathway generates two subsets – Langerhans cells (LCs), which are found in stratified epithelia such as the skin, and interstitial DCs, which are found in all other tissues [31]. The lymphoid pathway generates plasmacytoid DCs (pDCs), which secrete large amounts of IFN-α/β after viral infection [32, 33]. Each of the three DC subsets express a unique lectin [34]. LCs express Langerin, critical to the formation of Birbeck granules [35, 36]. The intDCs express DC-SIGN, involved in the interactions with T cells, DC migration, but also utilized by pathogens, for example HIV, to hijack the immune system [37–39]. pDCs express yet another lection BDCA2 [40, 41]. Toll receptors are also differentially expressed. For instance Toll 9 (a receptor for demethylated DNA) is expressed only by pDCs [42]. Such differential expression of surface molecules may permit specific *in vivo* targeting of DC subsets for induction of a desired type of immune responses, as recently demonstrated in mice by targeting DEC-205 [43, 44].

DCs and their precursors show remarkable functional plasticity. For example, pDCs form a first barrier to the expansion of intruding viruses, thereby acting, through the release of type I interferon, as part of the innate immune response. Subsequently, these cells differentiate into DCs that can present antigens to T cells, thus acting as members of the adaptive immune system [45, 46]. Monocytes

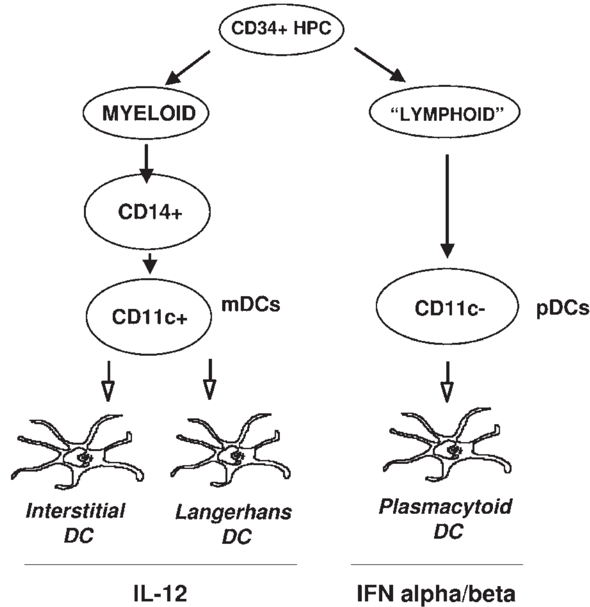

Fig. 52.1 Subsets of human dendritic cells. Blood DCs, mobilized by FLT3 ligand, contain both CD11c$^+$ myeloid DCs and plasmacytoid DCs. Most studies of DCs so far have been carried out with DCs made by culturing monocytes with GM-CSF and IL-4, a simple procedure that yields a homogenous population of DCs. These preparations contain cells that resemble interstitial DCs and are devoid of LCs. These DCs are immature and require exogenous factors for maturation. DCs can also be generated by culturing CD34$^+$ HPCs or blood monocytes with GM-CSF and TNF. In this way, two DC subsets can be obtained: LCs that may have improved efficacy in eliciting CTL, and interstitial DCs that resemble monocyte-derived DC. Adding IL-4 to CD34$^+$ HPC cultures with GM-CSF/TNF inhibits LC-differentiation.

can differentiate into either macrophages, which act as scavengers, or DCs that induce specific immune responses [47, 48]. Different cytokines skew the *in vitro* differentiation of monocytes into DCs with different phenotypes and function (Fig. 52.2). Thus, when activated (for example by GM-CSF) monocytes encounter IL-4 they will yield IL4-DCs [27, 28, 49]. By contrast, after encounter with IFN-α/β, TNF or IL-15, activated monocytes will differentiate into IFN-DCs [50–53], TNF-DCs [54] or IL15-DCs [55], respectively. Whether all interstitial DCs are derived *in vivo* from monocytes remains to be established. However, myeloid DCs isolated from human blood also give rise to different DCs upon exposure to different cytokines. Each of these DC subsets has common as well as unique biological functions, determined by a unique combination of cell-surface molecules and cytokines. For example, whereas IL4-DCs are a homologous population of immature cells devoid of LCs, TNF-DCs are heterogeneous and include both CD1a$^+$ LCs and CD14$^+$ interstitial DCs [54]. *In vitro* experiments showed that LCs and interstitial DCs generated in cultures of CD34$^+$ hematopoietic progenitors differ in their ca-

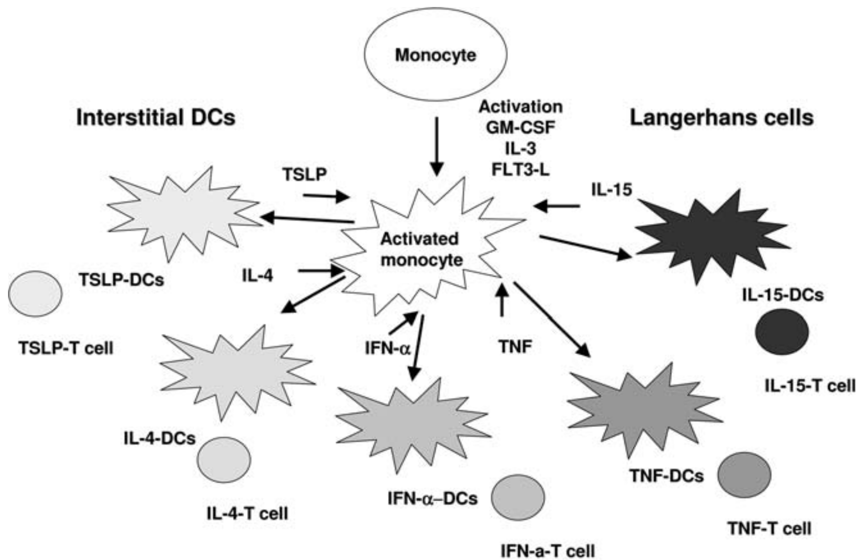

Fig. 52.2 Plasticity of monocyte-derived DCs. Activated monocytes will yield different DCs upon encounter with different cytokines. These different DCs will yield immune effectors, for example T cells, with distinct functions leading to different immune responses. For example, IL-15 DCs are remarkably more efficient in priming and maturation of rare antigen-specific CTLs as compared to IL-4 DCs. TSLP-DCs induce $CD4^+$ T cells to differentiate into pro-inflammatory Type 2 cells secreting large amounts of IL-13 and TNF [128] while IFN-DCs induce $CD4^+$ T cells to secrete IFN-gamma and IL-10.

pacity to activate lymphocytes: interstitial DCs induce the differentiation of naïve B cells into immunoglobulin-secreting plasma cells [10, 31], whereas LCs seem to be particularly efficient activators of cytotoxic $CD8^+$ T cells. They also differ in the cytokines that they secrete: only interstitial DCs produce IL-10; and their enzymatic activity [10, 31], which might be fundamental for the selection of peptides that will be presented to T cells. Indeed, different enzymes are likely to degrade a given antigen into different peptide repertoires, as recently shown for HIV nef protein [56]. This will lead to different sets of MHC/peptide complexes being presented and to distinct antigen-specific T-cell repertoires. Thus, these unique DCs are likely to yield unique immune effectors, therefore allowing the broad immune response necessary to combat mutating tumors.

52.3.2
Distinct Dendritic Cell Subsets Induce Distinct Types of Immune Response

DCs play a critical role in determining the type of response that is induced. There is evidence that either polarized DCs or distinct DC subsets may provide T cells with different signals that determine the class of immune response (Type 1 versus Type 2) [30]. Thus, in mice, splenic $CD8\alpha^+$ DCs prime naïve $CD4^+$ T cells to make

Th1 cytokines in a process involving IL-12, whereas splenic CD8α^- DCs prime naïve CD4$^+$ T cells to make Th2 cytokines [57, 58]. Furthermore, different signals can induce different T-cell polarization by the same DCs, as shown by the induction of IL-12 production and Th1-cell polarization when DCs are activated with *E. coli* LPS, but no IL-12 production and Th2-cell polarization when DCs are exposed to LPS from *P. gingivalis* [59]. In humans, CD40-ligand (CD40-L)-activated monocyte-derived DCs prime Th1 responses through an IL-12-dependent mechanism, whereas pDCs activated with IL-3 and CD40-L have been shown to secrete negligible amounts of IL-12 and prime Th2 responses [60]. Thus, both the type of DC subset and the activation signals to which DCs are exposed are important for T-cell polarization. This is an important parameter in vaccination against cancer, as Type 1 immunity with IFN-γ secretion is desired, whereas Type 2 immunity with IL-4 secretion, or production of IL-10, is considered deleterious.

52.3.3 Dendritic Cells and Immune Tolerance

Dendritic cells can induce and maintain immune tolerance [25], both central and peripheral. Central tolerance depends on mature thymic DCs, which are essential for the deletion of newly generated T cells with a receptor that recognizes self components [61]. However, central tolerance might not be effective for all antigens. Furthermore, many self antigens may not access the thymus and others are only expressed later in life. Hence, the need for peripheral tolerance, which occurs in lymphoid organs and is mediated by immature DCs. Immature DCs present tissue antigens to T cells in the absence of appropriate co-stimulation, leading to T-cell anergy or deletion [25], or the development of IL-10-producing, regulatory T cells [62, 63]. The groups of Nussenzweig and Steinman have elegantly shown that fusion proteins targeted to immature DCs lead to antigen-specific tolerance induction [43]. By contrast, concomitant activation of the DCs with CD40-specific antibody results in a potent immune response as DCs are induced to express a large number of co-stimulatory molecules [44]. However, mature DCs might also contribute to peripheral tolerance by promoting the expansion of naturally occurring suppressor T cells [64], as discussed later. Therefore, the biology of DCs offers several targets for the control of cellular immunity.

52.4 Dendritic Cells as Cancer Vaccines

52.4.1 Dendritic Cell Subsets

The discovery that GM-CSF and IL-4 can differentiate monocytes into immature DCs [27, 28, 49] has allowed major progress in our understanding of DC biology and function. Several groups have now used IL4-DCs as vaccines [14] following pi-

oneering clinical studies in patients with metastatic melanoma by Nestle et al. [65] (using tumor-lysate-loaded DCs) and by Schuler and colleagues [66] (using melanoma-peptide-loaded DCs). However, recent discoveries point to some alternatives to the classical way of generating DCs. For example, melanoma-peptide-pulsed IL15-DCs are much more efficient than IL4-DCs for the induction of antigen-specific CTL differentiation *in vitro*, whereas their ability to stimulate $CD4^+$ T cells is comparable [55]. Also, IFN-α-DCs generated in three-day cultures are efficient for the induction of specific immunity [52]. Thus, the immunogenicity of these distinct DC vaccines needs to be tested *in vivo* in clinical studies.

Monocytes are not the only source of DC precursors/progenitors that have been used in clinical studies. Blood DCs loaded with specific idiotype protein have been used as vaccines in patients with follicular B-cell lymphoma [67], where immune and clinical responses were observed. Similarly, blood DCs loaded *ex vivo* with a recombinant fusion protein consisting of prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor are being used in patients with prostate cancer [68]. Furthermore, Fong et al. enriched blood for DCs using FLT3 ligand and demonstrated the induction of immune and some clinical responses in vaccinated patients [69]. We have vaccinated patients with metastatic melanoma with antigen-loaded DCs derived from $CD34^+$ hematopoietic progenitor cells (CD34-DCs) [70]. CD34-DC vaccination elicited melanoma-specific immunity and patients who survived longer were those who mounted immunity to >2 melanoma antigens. These results justify the design of larger follow-up studies with a range of different DC vaccines to assess their immunological and clinical efficacy. Therefore, we feel that the proposal for standardization of DC vaccination protocols in cancer [71] might be premature, as the optimal type of DC to be used for vaccination still remains to be determined.

52.4.2
Dendritic Cell Maturation

Immature DCs are predominantly antigen capturing cells while mature DCs are predominantly antigen presenting cells. DC maturation *in vivo* is intimately linked with their migration from the peripheral tissue to the draining lymphoid organs and is associated with several coordinated events including: (1) loss of endocytic/phagocytic receptors; (2) upregulation of co-stimulatory molecules CD40, CD58, CD80, CD86; (3) change in morphology, (4) shift in lysosomal compartments with down-regulation of CD68 and up-regulation of DC-LAMP; and (5) change in class II MHC compartments. Immature DCs are weak immunogens and as discussed above can be tolerogenic. Accordingly, intranodal injection of immature DCs generated from monocytes with GM-CSF and IL-4 does not lead to significant immune responses, in contrast to the intranodal injection of mature IL-4 DCs in the same patient [72]. Mature IL-4 DCs induce functionally superior $CD8^+$ T cells and polarize $CD4^+$ T cells towards IFN-γ production [73]. Yet, the current "gold standard" method to induce DC maturation with a cocktail of pro-inflammatory cytokines (IL-1β, TNF, IL-6 and PGE2) [74] needs to be examined in parallel with other

stimuli. Indeed, a combination of IL-1β and TNF with type I (IFN-α) and II (IFN-γ) interferons seems to yield more potent DCs in terms of IL-12 secretion and induction of tumor-specific CTLs *in vitro* [75]. It will therefore be important to identify stimuli that trigger a desired DC maturation program – leading to induction of tumor-specific CTLs but limited or no induction of suppressor T cells – in the various human DC subsets. For example, Toll-like receptors ligands could be used to mimic the "natural" pathogen-induced maturation pathways of DCs. These, together with a T cell-like signal delivered through CD40, may enhance DC function [76]. Indeed, TLR-mediated signals are involved in the control of $CD4^+$ T-cell activation [77] and, for example, DCs loaded with a heart-specific self-peptide induce $CD4^+$ T-cell-mediated myocarditis in nontransgenic mice if activated through both CD40 and TLRs [78]. Interestingly, Toll receptors are differentially expressed by distinct DC subsets. For example, Toll 9 (a receptor for demethylated DNA) is expressed only by pDCs while mDCs preferentially express Toll 2 and 4 (receptors for bacterial products such as peptidoglycan and lipopolysaccharide, respectively) [42]. Such differential expression may confer distinct maturation signals yielding distinct type of immune responses.

52.4.3
Dendritic Cell Migration

The number of DCs injected, and ultimately the number of DCs that migrate into draining lymph nodes, is also likely to affect T-cell priming. Monitoring the *in vivo* migration of labeled DCs in patients showed that only a small fraction (<1%) of intradermally injected DCs migrated rapidly to the regional lymphatics [79]. DC migration was improved in a mouse study by conditioning the injection site with TNF, which significantly increased DC migration to the draining lymph nodes and the magnitude of the $CD4^+$ T-cell response [80]. Furthermore, distinct maturation/activation signals, for example PGE2 [81, 82], may induce the preferential expression of CCR7 by DCs, hence increasing the capacity of the DCs to respond to appropriate ligands such as CCL19 and CCL21 expressed in lymphatic vessels and secondary lymphoid organs [83].

Antigen-loaded DCs may prime T-cell responses regardless of the route of injection, but the quality of responses is affected, as shown by the induction of predominant Th1 responses after intradermal and intralymphatic administration, but unpolarized T-cell and antibody responses after intravenous administration [84]. Furthermore, recent studies in mice show that the induction of tissue-specific immunity is related to the tissue origin of DCs [85, 86]. Both intravenous and subcutaneous immunization with peptide-pulsed DCs in a mouse model of melanoma induced peptide-specific memory T cells in spleen and permitted the control of lung metastasis. However, whereas subcutaneous immunization also induced memory T cells in the lymph nodes allowing subsequent protection against subcutaneously growing tumors, intravenous immunization failed to do so [86]. Thus, DCs can prime T cells with different homing capacities. The consequence of such instructive role for the therapeutic vaccination with DCs is that, for example, intravenous

administration of a DC vaccine for melanoma would be unlikely to induce skin-homing effector cells.

Another important parameter that affects the number of DCs migrating to the draining lymphoid tissue and the resulting immune response is the frequency of vaccination. We have found in 18 patients with metastatic melanoma that four vaccinations over 6 weeks with melanoma peptide-loaded CD34-DCs result in an increase in the number of melanoma-specific $CD8^+$ T cells in the blood as documented by IFN-γ ELISPOT [70, 87] and CTL assay [88]. However, the melanoma-specific $CD8^+$ T-cell immunity in the blood was short-lived: all analyzed patients lost specific T cells detectable by direct ELISPOT and 4/9 patients lost all recall responses by two months after the last vaccination. Several explanations might be considered. T cells might migrate from the blood to peripheral tissue (tumor site) [89]. Alternatively, the four bi-weekly vaccinations might have provided too frequent antigen stimulation for optimal T-cell differentiation. Mouse and human studies of vaccination against infectious agents [90, 91] indicate that priming should be followed by a boost 4–6 weeks later for an optimal response. However, these rules may not apply to a chronic disease such as cancer. By analogy, chronic viral infections are associated with exhausted T cells owing to chronic antigen presentation [92, 93], and their reactivation through vaccination is likely to require different schedules.

52.4.4 Antigen Loading

Loading MHC class I and MHC class II molecules on DCs with peptides derived from defined antigens is the most commonly used strategy for DC vaccination [20, 94]. Although this technique is important for "proof of concept" studies, the use of peptides has limitations: restriction to a given HLA type; the limited number of well characterized tumor-associated antigens (TAA); the relatively rapid turnover of exogenous peptide-MHC complexes resulting in comparatively low antigen presentation at the time the DC arrive into draining lymph node after injection; and, the induction of a restricted repertoire of T-cell clones, thus limiting the ability of the immune system to control tumor antigen variation. Yet another level of complexity is brought about by the use of modified heteroclitic peptides. Some synthetic peptides, even those derived from immunodominant antigens, do not bind MHC class I molecules with high affinity, possibly explaining their limited immunogenicity *in vivo* [95]. Therefore, the generation of peptide analogs with increased affinity for MHC class I binding (heteroclitic peptides) could be used to improve immunogenicity [96, 97]. However, recent elegant studies in patients with malignant melanoma show that T cells elicited *in vivo* by vaccination with heteroclitic MART-1 and gp100 peptides show poor recognition of native peptide and less efficient tumor lysis compared with endogenous peptide melanoma-specific T cells [98]. The most likely explanation is that these peptides differ from naturally processed epitopes. Thus, loading DCs with total antigen preparations and allowing "natural" processing and epitope selection is expected to improve efficacy as well as to allow the generation of a diverse immune response involving many clones of

$CD4^+$ T cells and CTLs. These strategies include loading DCs with recombinant proteins, exosomes [99], viral vectors [100], plasmid DNA, RNA transfection [101], immune complexes [102] and antibodies specific for DC surface molecules [20, 103]. Another technique involves exploiting the capacity of DCs to present peptides from phagocytosed dead tumor cells on MHC class I molecules (as well as class II molecules), which is known as cross-priming [104, 105]. Indeed, DCs cultured with killed allogeneic melanoma, prostate and breast cancer cell lines prime naïve $CD8^+$ T cells against tumor antigens *in vitro* [105, 106]. We have now vaccinated 20 patients with metastatic melanoma with autologous monocyte-derived DCs previously exposed to a killed allogeneic melanoma cell line (8 vaccines on a monthly basis). Vaccination has proved to be safe (no autoimmunity or other adverse events) and has led to the induction of melanoma-specific T-cell immunity. In two patients, this has resulted in long-lasting tumor regression (J.B. and A.K.P., unpublished observations). These results warrant larger clinical studies.

52.5 Regulatory/suppressor Mechanisms

A major obstacle to the success of cancer vaccines including DCs might be the presence of regulatory/suppressor T cells and the demonstration that DCs regulate their expansion (Table 52.2). Two broad subsets of $CD4^+$ T cells with regulatory function have been characterized. Naturally occurring $CD4^+CD25^+$ suppressor T cells are naturally "anergic" and require stimulation through the TCR for their suppressive function [107, 108], which is cell mediated and independent of IL-10 or TGF-β. Mature DCs facilitate the expansion of these cells *in vivo* in mice [64]. Induced regulatory T cells [109–111] are derived from $CD4^+CD25^-$ T cells and mediate their effects through IL-10 and TGF-β. The differentiation of these T cells seems to be controlled by immature DCs [62]. A large body of experimental evidence shows that these T cells suppress anti-tumor immunity and that their removal allows tumor eradication [112, 113]. An increased frequency of $CD4^+CD25^+$

Tab. 52.2 Regulatory/suppressor mechanisms that may hamper vaccine efficacy.

Naturally occurring $CD4^+CD25+$ suppressor T cells	Cell-mediated suppression independent of IL-10 and/or TGF-β; regulated by mature DCs
Induced regulatory T cells	Cytokine-mediated suppression through IL-10 and/or TGF-β; regulated by immature DCs
NKT cells	Cytokine-mediated suppression through IL-13
Tumor-specific IFN-gamma-secreting T cells?	Immuno-editing and selection of escape variants
Vaccine induced B cells?	Cytokine-mediated regulation through IL-4, IL-6 and IL-10; competition with DCs for antigen uptake

T cells has been observed in the blood and tissues of patients with cancer [114–116]. However, other cells with suppressive function exist that might also limit vaccine efficacy, for example NK-T cells through their production of IL-13 [117].

While many patients show immune responses to control antigens on DCs, for example memory CD8$^+$ T-cell responses to antigens derived from Influenza virus or primary CD4$^+$ T-cell responses to KLH protein, some fail to mount an immune response to tumor antigens. These observations raise a question whether the failure of DCs to induce tumor-specific therapeutic immunity in these patients is related to the DC vaccine or to host factors? A possible explanation could be that tumor-specific T cells from these patients cannot be primed *in vivo*. *In vitro* experiments show, however, that in most of the patients, the T cells can be primed to differentiate into CTLs with specificity for multiple melanoma antigens [105]. Even if the T cells are primed *in vivo*, the tumor microenvironment in patients with progressive disease may be more aggressive and lead to the elimination of tumor-specific T cells *in situ*. Also, patients with progressive disease may have an increased number of regulatory/suppressor cells that do not allow efficient *in vivo* priming of CD8$^+$ T cells or their function. These immune effectors are likely to be differentially regulated by distinct DC subsets. Therefore, it is important to study how human DC subsets, generated either from distinct lineages, such as pDCs versus mDCs, or as a result of environmental regulation, such as monocytes cultured with IFN-α [52, 53] versus those cultured with GM-CSF and IL-4 [27, 28], modulate immune effectors.

Naturally occurring CD4$^+$CD25$^+$ suppressor T cells may be controlled by pre-treatment of patients with drugs that can eliminate and/or control these cells, meaning that DC vaccination may be more effective when combined with other therapies. Studies in the late 1970s and early 1980s showed in animal models that cytostatic drugs (for example, cyclophosphamide) facilitate adoptive immunotherapy for tumors [118]. The proposed mechanism was the elimination of suppressor T cells [118]. Recent data showing improved outcomes of vaccination with DCs in myeloablated animals [7, 8] reinforce this concept and indicate that controlled "immune ablation" may improve the clinical efficacy of DC vaccination trials in cancer. Besides the elimination of suppressor T cells, the mechanism may also involve the elimination of pre-existing memory T cells, which might not be of the most effective phenotype (for example, Th2). Thus, pre-treatment of patients with metastatic cancer with cyclophosphamide prior to vaccination with DCs might significantly improve DC vaccine efficacy.

52.6 Immunological and Clinical Efficacy

52.6.1 Immunological Efficacy

The efficacy of vaccines against cancer can be studied by detailed measurement of elicited T-cell responses in the blood. Anti-tumor immunity has been classically

measured by the quantity of tumor-antigen specific $CD8^+$ T cells [119]. However, there is no defined threshold for how many T cells are sufficient to induce tumor regression.

The discrepancy between the immune and clinical responses observed in cancer vaccination trials might be related to the breadth and quality of elicited T-cell immunity. Indeed, our own results in patients with metastatic melanoma vaccinated with CD34-DCs pulsed with multiple melanoma peptides show a better clinical outcome in patients who mounted immunity to several tumor antigens as measured in the blood [70]. Furthermore, the elicited tumor antigen-specific T cells should be capable of cytokine production, proliferation upon antigen re-exposure, migration to the tumor site and CTL function [98].

Other immune effectors including $CD4^+$ T cells, NKT and NK cells, and B cells also need to be taken into account. In particular, $CD4^+$ T cells seem to be fundamental for priming long-lived $CD8^+$ T-cell memory [120–122]. In fact, the lack of $CD4^+$ T-cell activation in peptide-vaccination strategies might explain their limited efficacy. Although a large number of circulating effector $CD8^+$ T cells might be elicited by such vaccines, in the absence of $CD4^+$ T-cell help, their quality might be compromised and the establishment of specific $CD8^+$ T-cell memory is unlikely [123]. The induction of NKT cells, which kill a wide spectrum of tumor cells [124], or NK cells, which recognize MHC-class-I-deficient tumor cells [125], could be desirable, yet caution must be taken with regard to the cytokines that they produce. For example, IL-13-producing NKT cells may inhibit CTL-mediated tumor elimination and favor tumor progression [117]. Finally, B cells may inhibit the induction of T cell-mediated tumor immunity by competing with DCs for uptake of tumor derived antigens [126], or through cytokine secretion [127]. Yet, in the active immunization setting, there may also be desirable humoral responses and we certainly need to learn more about the types of humoral immunity that are induced by the different DC subsets. Thus, there is a need for comprehensive, multi-parameter immunomonitoring to truly assess the level of tumor immunity. This comprehensive approach should also incorporate the analysis of T cells present at the tumor site.

52.6.2
Clinical Efficacy

Are DC vaccines better than other types of vaccine? Vaccination with antigen-loaded DCs showed 9.5% tumor regression – 11/116 patients responded in six different clinical trials – compared with a maximum of 4.6% for other protocols. Although still limited, these early outcomes warrant further exploration to establish the therapeutic value of vaccination with DCs.

The definition of clinical endpoints, and hence the measures that are used to assess vaccine efficacy, need to be revisited. Since cancer is a chronic disease, prolonged survival and good quality of life might be considered a therapeutic success and of benefit to the patient. Therefore, while critically assessing different therapies, we should be careful not to pre-maturely dismiss therapies that do not lead to a high rate of objective tumor regression [17]. Furthermore, it might be considered

unrealistic to expect even the most efficient immune responses to eliminate the total tumor burden in a patient with stage IV cancer.

52.7 Conclusions

DCs are an attractive target for therapeutic manipulation of the immune system to enhance otherwise insufficient immune responses to tumor antigens. However, the complexity of the DC system requires rational manipulation of DCs to achieve protective or therapeutic immunity. Thus, further research is needed to analyze the immune responses induced in patients by distinct *ex vivo* generated DC subsets activated via different pathways. The ultimate *ex vivo*-generated DC vaccine will be heterogeneous and composed of several subsets, each of which will target a specific immune effector. These *ex vivo* strategies should help to identify the parameters for DC targeting *in vivo*, which represents the next step in the development of DC-based vaccination.

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53
Renal Cell Carcinoma

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53.1
Dendritic Cells and Cancer Immunosurveillance

During the last decade the number of PubMed search results using the search term "dendritic cells/immunology" has increased approximately 10-fold. The enhanced interest in DCs has been accompanied by a boost of the research in basic and clinical immunology and by a re-emergence of interest in the tumor immunosurveillance concept. After periods of considerable scepticism, today the issue is not if – but, rather, how – tumor immunosurveillance works to prevent the development of cancer in the immunocompetent host [1]. Lymphocytes of the innate (NK, NKT cells and γδ T cells) and adaptive immune system (αβ T cells) are required to prevent the development of both chemically-induced and spontaneous tumors. The two critical functions of these lymphocytes in tumor cell eradication are the ability to produce IFN-γ and the ability to kill.

The fact that DCs activate and/or modulate all these lymphocyte subsets has stimulated great interest in DC-based immunotherapeutic approaches to control and/or eliminate human tumors. A serious and lengthy attempt to validate the concept of DC-based immunotherapy of human tumors in clinical trials has recently been initiated [2].

53.2
Renal Cell Carcinoma

Renal cell carcinoma (RCC) collectively refers to a heterogenous group of neoplasms originating from proximal tubular epithelial cells. RCC accounts for 2–3% of all malignancies and is the most common cancer of the kidney [3, 4] (Fig. 53.1). RCC affects more than 30 000 individuals each year in Europe and has a peak incidence in the 5th and 6th decade of life. About one third of the patients has metastatic disease at diagnosis and 30–50% of initially localized RCCs eventually metas-

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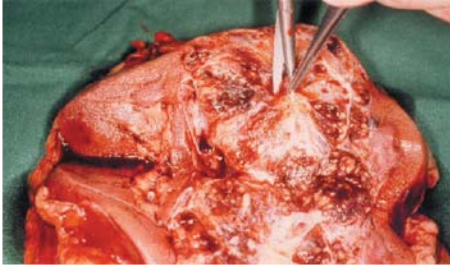

Fig. 53.1 Renal cell carcinoma: primary tumor in the kidney.

tasize. RCC is virtually resistant to chemotherapy and hormone therapy. Patients with metastatic RCC have a median survival time of 10.2 months reflecting the lack of effective treatment of metastatic disease [5].

The frequent leukocytic infiltration of RCC tissue reflects an ongoing or past interaction between the tumor and the immune system [6]. Moreover, occasional regressions and a certain responsiveness to systemic cytokine therapy [7] have strengthened the view that RCC is an immunogenic tumor and that the development of effective immunologically based therapies would be desirable.

53.3 Immunotherapy of Renal Cell Carcinoma

Failure of conventional approaches, such as chemotherapy or hormone therapy, has led to the development of immunotherapeutic regimes for the treatment of metastatic RCC. In strongly varying protocols, cytokines, lymphocytes and modified tumor cells have been applied to mobilize the immune system against the growing tumor. Nonspecific immunotherapy has administered cytokines like interferon-α [8] or interleukin-2 alone [9, 10] or in combination [7]. Cytokine-based immunotherapy is limited by sometimes severe side effects, barely tolerable by the mostly elderly patients. The passive transfer of tumor-infiltrating lymphocytes (TIL) or lymphokine-activated killer (LAK) cells activated and expanded *in vitro* turned out to be ineffective [10]. In the active, specific immunotherapy the immune system is prompted to specifically target and attack distinct tumor-associated antigens. This approach is a vaccination by nature, although it is therapeutic (as opposed to prophylactic vaccination such as the known vaccinations which serve to prevent infectious diseases). The advantage of the active, specific immunotherapy is in its specificity and in the development of an immunologic memory that should be able to prevent recurrence of the disease. Within this category, gene-modified tumor cells producing GM-CSF have been used to vaccinate RCC patients against their tumors [11]. An intriguing finding of this trial was that biopsies of intradermal sites of injection with GM-CSF gene-modified RCC cells contained distinctive DC infiltrates pointing toward an important role of these cells in the induction of tumor immunity. DCs are indeed highly attractive for the active specific immunotherapy since their natural function is to induce antigen-specific immunity [12].

53.4 Dendritic Cell-based Immunotherapy of Renal Cell Carcinoma

53.4.1 The Two-step Culture System

The discovery that DCs can be cultured in sufficient numbers from abundant circulating monocytes paved the way for clinical research. A frequently used protocol is based on a two-step culture system [13, 14]: in the first step monocytes are differentiated into immature DCs in the presence of GM-CSF and IL-4. Then, inflammatory stimuli induce a maturation step, which is characterized by a re-programming that enhances the immunostimulatory potential of the DCs. In our hands, this culture system turned out to be feasible and reliable both in healthy individuals [15] and in RCC patients [16].

53.4.2 Generation of Clinical Grade Dendritic Cells

DCs generated for clinical immunotherapy must be considered a pharmaceutical product, which requires the standards of good manufacturing practice (GMP). Although the laboratories in a University environment do not usually meet these criteria, considerable attempts have been made to approximate the requested standards. All work is performed in a completely separated cell culture laboratory, which is routinely subjected to disinfection and validation protocols. People working in this laboratory wears sterile coats as well as sterile headpieces, gloves and masks.

Our current method [17] starts with leukapheresis performed with the Cobe Spectra cell separator. $CD14^+$ monocytes are then separated from the leukapheresis product by positive selection using the MACS Technology from Miltenyi Biotec (CD14 Reagent and the CliniMACS Instrument). The purified $CD14^+$ cells (50×10^6 in 50 ml) are cultured in 162 cm^2 cell culture flasks in AIM-V culture medium containing 1% heat-inactivated human AB plasma as well as a combination of recombinant human GM-CSF and recombinant human IL-4. After 2 days of culture, 50 ml of fresh medium containing supplements are added. Day-5 moDCs are harvested and frozen in liquid nitrogen using a standard protocol (50% AIM-V, 40% human AB plasma, 10% DMSO). Two days before vaccination, immature moDCs are thawed, counted and 18×10^6 cells are re-plated in 6-well plates at 1.8×10^6 cells per well in 6 ml of fresh medium containing cytokines as well as 1% heat-inactivated human AB plasma.

In a recent trial [18], the mean number of PBMCs obtained by leukapheresis was 87×10^8 ranging from $60–140\times10^8$. The mean number of $CD14^+$ cells was 10×10^8 ranging from $7–16\times10^8$ resulting in a mean number of 4.6×10^8 ($2–9\ 10^8$) immature DCs. On average, 66.7% of the plated immature DCs were recovered after antigen loading and maturation.

In both trials so far performed in our department [18, 19], DC maturation was induced by a combination of TNF-α, IL-1β, IL-6 and PGE2 [20, 21]. Although this

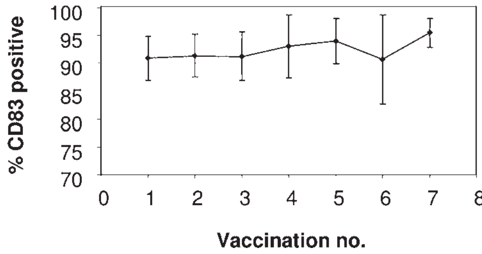

Fig. 53.2 Reliable generation of $CD83^+$ mature dendritic cells.

combination may not be the ultimate cocktail for the maturation of fully activated DCs, it has nevertheless been extremely reliable for the maturation of $CD83^+$ DCs. Fig. 53.2 depicts mean percentages of $CD83^+$ cells in 61 vaccine preparations and demonstrates the consistently high percentage of $CD83^+$ cells throughout the treatment course.

In this context it may be important to note that effects usually attributed to PGE2 may in fact be mediated by PGA2. PGA2 is a cyclopentenone derivative of PGE2 and is rapidly generated by non-enzymatic dehydration of PGE2 *in vitro*. We recently demonstrated that PGA2 is nearly as potent as PGE2 in enhancing the TNF-α induced maturation of human moDCs [48]. Despite the chemical difference between PGE2 and PGA2 (cyclopentane versus cyclopentenone ring) and the reported opposing effects on inflammation (pro- versus anti-inflammatory), they likewise enhanced phenotypic moDC maturation as well as the maturation-associated increase in migratory and T-cell stimulatory capacity. Cytokine profiles established from T-cell cultures stimulated with either PGE2 or PGA2-treated moDCs were consistent with enhanced Th1 type immunity.

Sterility tests included the incubation of the test sample in both solid and fluid media to detect the growth of contaminating bacteria (anaerobic and aerobic, gram-negative, gram-positive) and fungi (yeast and mold), respectively. All cell cultures as well as the final vaccine consisting of tumor lysate-pulsed, $CD83^+$ DCs were subjected to sterility testing. All 399 vaccines prepared to date remained negative.

53.4.3
Clinical Trials of Dendritic Cells in Renal Cell Carcinoma Patients

The clinical trials with DCs performed so far in RCC patients are heterogenous [18, 19, 22-28]. They differ in the source of the DCs (autologous versus allogeneic), in the maturation status (immature versus mature), in the antigen formulation used for dendritic cell loading (tumor lysate with or without helper antigen, tumor–dendritic cell fusion, tumor RNA transfection) as well as in the use of a helper antigen. Comparison is difficult and definitive conclusions are impossible. Table 53.1 summarizes some basic data from the published trials.

Nevertheless, some interesting observations have been made. The available data suggest that mature DCs should be preferred in future trials. This is in accordance with a well accepted concept of basic immunology that has recently emerged, in

Tab. 53.1 Clinical studies with dendritic cells in human metastatic renal cell carcinoma.

DCs	*Antigens*	*Route*	*Clinical response*	*Reference*
Autologous mature moDCs	tumor lysate, KLH	i.v, i.d.	2 CR, 1 PR, 7 SD, 17 PD	19
Autologous mature moDCs	tumor lysate, KLH	intra-, perinodal	1 PR, 7 SD, 7 PD	22
Autologous immature moDCs	tumor lysate	i.d.	1 PR, 3 SD, 8 PD	24
Autologous immature moDCs	tumor lysate, KLH	i.d. + IL-2	8 SD, 4 PD	25
Autologous semi-mature moDCs	tumor RNA	i.v., i.d.	7 SD, 3 PD	26
Allogeneic mature moDCs	tumor lysate, KLH	i.v., id. ± Cy	2 MR, 3 SD, 13 PD	18
Allogeneic immature moDCs	tumor lysate, KLH i.d. injection	i.d.	2 SD, 3 PD	27
Allogeneic immature moDCs	moDC – tumor cell fusion	i.d.	4 SD, 8 PD	23
Autologous immature moDCs	moDC – tumor cell fusion, KLH	s.c.	5 SD, 11 PD	28

moDCs, monocyte-derived DCs; KLH, keyhole limpet hemocyanin; i.v., intravenous; i.d., intradermal; IL-2, interleukin-2; Cy, cyclophosamide; CR, complete resonse; PR, partial response; MR, mixed response; SD, stable disease; PD, progressive disease.

which the maturation state of DCs swings the decision between tolerance and immunity [29]. While immature DCs maintain tolerance by silencing antigen-specific T lymphocytes or by actively converting them into regulatory T cells [30], mature DCs promote immunity.

The immunogenicity of mature moDCs, i.e. the ability of moDCs charged with antigens *ex vivo* to induce antigen-specific responses *in vivo*, has repetitively been confirmed. For this purpose immunogenic xeno-antigens have been loaded onto moDCs and various methods of cellular immunology have been used to monitor the elicited immune responses. A frequently used control antigen is keyhole limpet hemocyanin (KLH). In our own studies, we found that moDCs charged with KLH can induce vigorous Th1 type immune responses in patients with metastatic RCC [19, 31-34].

A xeno-antigen such as KLH loaded onto DCs may serve another important purpose. As a helper antigen it will lead to activation of potent $CD4^+$ helper T cells which create a cytokine milieu that may favor the activation of comparatively weak tumor-reactive T cells. In addition, triggering of CD40 on DCs by CD40 ligand expressed by activated helper T cells leads to full activation of DCs and to licensing of the DCs for the activation of $CD8^+$ cytotoxic T cells [35]. *In vitro* models have demonstrated that KLH can indeed augment DC-induced antitumor immune respons-

es [36, 37]. Moreover, in a model of DC-induced autoimmune myocarditis the importance of CD40 triggering for the evolvement of full DC activity has recently been confirmed [38].

A number of tumor-associated antigens has been described for RCC including G250, Her2/neu, or oncofetal antigen [39-41]. However, broadly expressed antigens with relevance for tumor rejection are still rare. This has led to the use of tumor lysate as a source of tumor antigens. While tumor lysate represents a broad spectrum of potentially relevant tumor antigens, little evidence is available that DCs can indeed "extract" antigens from tumor cell lysates for subsequent processing and presentation indicating that there is a need for standardized antigen preparations and for validation data demonstrating that DCs can handle the antigens contained in these preparations.

In addition to autologous DCs, which are derived from the patient, allogeneic DCs generated from healthy donors have also been tested. Allogeneic DCs were either loaded with tumor lysate [18, 27] or fused with tumor cells (autologous or allogeneic) [23]. In the allogeneic setting, disease stabilization occurred in some patients but no tumor regressions were observed and immunity against control antigens could hardly be induced [18]. These findings confirm that the HLA barrier prevents the induction of immunity and suggests that antigen transfer from the allogeneic DCs to autologous DCs *in vivo* may be inefficient. The DC-tumor cell hybrid approach still suffers from a relatively poor fusion efficacy which was 14.3 ± 7.8% in one study [23]. Under conditions of poor fusion efficacy the hybrid approach resembles the lysate approach, since DCs may in principle take up and present antigens during coculture with the tumor cells.

53.5
Adjuvant Immunotherapy of Organ Confined Renal Cell Carcinoma after Partial or Radical Nephrectomy

Up to 50% of initially localized RCCs eventually progress and develop distant metastases indicating the need for an effective adjuvant treatment. In a recent randomized controlled trial an autologous renal tumor cell vaccine was tested in an adjuvant setting [42]. After nephrectomy, patients received in 4-week intervals six intradermal injections of lysates generated from autologous IFN-γ treated RCC cells. The primary endpoint was progression-free survival. 5-year progression-free survival rate for patients at all tumor stages was 77.4% in the vaccine group (177 patients) and 67.8% in the control group (202 patients) ($p = 0.0204$). These data suggest that antitumor vaccination may be useful to delay or prevent tumor recurrence after nephrectomy in patients with organ-confined disease. Tumor lysates exhibit low inherent immunogenicity. To increase immunogenicity tumor cells were treated with IFN-γ prior to lysis. Intradermal injection of the lysate increased the chance that tumor antigens are taken up by DCs which are abundant in the upper layers of the skin. Thus, antitumor immunity induced by this autologous renal tumor cell vaccine is likely to be mediated by DCs. In future studies it

would be attractive to test the potency of mature DCs generated *in vitro* in an adjuvant setting.

53.6
Patient Selection in Future Trials

Another important issue of immunotherapy is patient selection. Immunotherapy depends on a more or less intact immune system. A useful commercial test to assess the patient's immune status was the Multitest CMI kit from Merieux which tested for delayed-type hypersensitivity (DTH) against recall antigens. This test indicated that the immune system in a substantial proportion of the patients with metastatic RCC is indeed compromised [18, 19] and that such non-responsiveness is predictive of shortened survival (own unpublished observation). On the other hand, this test has been used to demonstrate that continued dendritic cell vaccination can lead to an increase in reactivity against recall antigens indicating that treatment with DCs can generally improve the functions of the immune system [22, 23]. Unfortunately, this test has been withdrawn from the market.

Motzer et al. described five pretreatment clinical features predicitve of survival: performance status, serum lactate dehydrogenase, hemoglobin level, corrected serum calcium, and nephrectomy status [4]. In addition, good nutritional state [43] may be used for patient selection.

The increasing efforts of genomics and proteomics [44] as well as of metabolomics [45] may also be helpful to better classify the heterogenous family of RCCs. In addition such attempts may help to establish surrogate markers of immune status and overall immunologic responsiveness which may then also be used to select patients suitable for immunotherapy. Metabolic profiles associated with immune response or immune deviation may further complement the current methodology of immune monitoring.

53.7
Adverse Effects – Quality of Life

An undeniable advantage of DC therapy is the virtual absence of adverse effects. At dosages of up to 1×10^7 cells given as intradermal injection side effects were either restricted to mild flue like symptoms or absent. In contrast, cytokine therapy using interleukin-2, interferon-α or combinations can have dramatic side effects barely tolerable by the mostly elderly patients. Our group has started to assess quality of life in patients undergoing DC therapy. Using the standardized, validated quality of life questionnaire QLQ-C30 provided by the European Organization for Research and Treatment of Cancer (EORTC), we found that quality of life remains high during treatment and may even be increased in some patients (own unpublished observations). When DC therapy is compared to more conventional treatments such as cytokine therapy or chemotherapy quality of life assessments

should be included since patient benefit eventually consists of extended survival accompanied by unimpaired quality of life.

53.8
Concluding Remarks

Figdor and colleagues have recently proposed minimum quality criteria for the design of clinical trials with DCs as well as quality criteria for the dendritic cell vaccine providing useful guide lines for future trials [46]. Following such guidelines would also allow comparison of different trials. In their recent viewpoint article in *Science* Steinman and Mellman emphasized that immunotherapy – to be successful – requires a broadening of basic research in humans [47]. Fundamental changes in infrastructure, funding mechanisms and eventually in the culture of the scientific community are required to bring immunology to medicine. Although currently restricted to cancer patients, the analysis of the human immune system should also help to improve therapies of other disorders associated with immune dysfunction. There is probably no biomedical discipline that has a greater potential for affecting human health than studies of the immune system.

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XX
Antigen Delivery

54
Crosspresentation and Loading of Tumor Antigens for Dendritic Cell Vaccination against Cancer

Madhav V. Dhodapkar

Protective immunity to tumors and pathogens involves the coordinate induction of both innate and adaptive T-cell immunity. Tumor cells however are generally poor antigen presenting cells (APCs), and are thought to be incapable of initiating T-cell immunity by themselves [1, 2]. Similarly, many of the pathogens do not directly infect APCs. It has therefore been hypothesized that the generation of T-cell immunity to these antigens depends on the acquisition and presentation of antigens derived from tumors or virus-infected cells by bone marrow-derived APCs [3]. Dendritic cells (DCs) are specialized APCs in that they have a remarkable capacity to acquire and present exogenous antigens from tumor or virally infected cells to elicit $CD4^+$ and $CD8^+$ killer T-cell responses [4]. This property of DCs to "crosspresent" exogenous antigens has been extensively studied, and insights into the biology of this process are reviewed elsewhere in this book. Although the exact contribution of crosspresentation of antigens for the generation of T-cell responses to pathogens and tumors *in vivo* is still debated [5, 6], this phenomenon provides new opportunities for loading of antigens on dendritic cells (DCs) for active immunization against cancer or pathogens. Here I will review the application of this emerging biology towards improving DC-mediated immunization of cancer in humans.

54.1
Approaches to Antigen Loading for Dendritic-cell-mediated Immunotherapy

The recognition of central role of DCs in the generation and regulation of T-cell immunity has led to attempts to target tumor antigens to DCs. This includes both the generation and antigen loading of DCs *ex vivo* prior to adoptive transfer, as well

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as targeting DCs directly *in situ*. Indeed, the importance of attention to DC biology is being increasingly appreciated to improve the efficacy of existing vaccine approaches [7].

Active immunization via adoptive transfer of DCs has been facilitated by the development of newer methods of generating DCs from blood monocytes or $CD34^+$ hematopoietic progenitors [8]. Advantages of *ex vivo* manipulation of DCs includes the control of DC quality (e.g. maturation status, DC subset), and expression of desired antigens. Many of the early studies of DC vaccination utilized DCs loaded with defined MHC I and/or MHC II restricted peptides; or proteins derived from tumor or viral antigens. These studies established the ability of these DCs to stimulate both CD4 and $CD8^+$ T-cell responses *in vivo*, both in healthy volunteers and cancer patients [9, 10]. However there are several caveats with the use of peptide pulsed DCs. For example, the longevity of the MHC-peptide complexes *in vivo* is unknown, affinity of peptides for various HLA molecules varies, peptide competition may affect immunogenicity, and the requirement of prior knowledge of HLA-restricted epitopes from tumor antigens, which limits application in many patients.

Several other approaches have also been attempted to load tumor antigens to DCs. These include the use of viral vectors, tumor-derived RNA, DNA, immune complexes (ICs), apoptotic tumor cells, tumor cell lysates, or heat shock proteins [8, 11]. Many of these methods are aimed at exploiting the ability of DCs to cross-present exogenous antigens. The use of genetically modified DCs is reviewed elsewhere in this book. Here I will review the approaches to loading DCs with antigens that take advantage of the ability of DCs to crosspresent tumor-derived antigens. Although several major gaps remain in our understanding of the mechanism of crosspresentation by DCs *in vivo* in humans, improved understanding of the process suggests several steps that might regulate the efficiency of crosspresentation by DCs.

54.2
Importance of Receptor-mediated Uptake to Crosspresentation

Dendritic cells can capture bacteria, viruses, dying cells, soluble proteins, and immune complexes through several mechanisms including phagocytosis, endocytosis and pinocytosis [12]. They also have an array of cell surface receptors for antigen uptake, many of which also function in signaling or cell–cell interactions. In some instances, such as dying cells, several receptors are likely to be involved. For others such as DEC-205, the natural ligands remain to be identified. A growing body of data suggests that the efficiency of antigen presentation is greatly influenced by receptor mediated uptake of antigens. This may in turn depend on the biology of the uptake receptor and its interactions with other molecules involved in antigen presentation.

54.3
Uptake of Dying Cells

In many human tumors, the antigenic targets expressed by tumor cells are not fully known. Even in the case of tumors wherein some of the antigens have been defined, it is possible that the antigenic profile in each patient's tumor will be specific to each patient [13]. These considerations have prompted the use of whole tumor cells, either cell lines or autologous tumor cells as sources of tumor antigens. Most studies to date have focused on the use of dying cells, using either irradiated tumor cells or lysates derived from these cells to load human DCs [14–17], although it is by no means clear that cell death is required for the access of cellular antigens to the crosspresentation pathway. Indeed, crosspresentation of live cellular material from both virally infected and tumor cells has been shown [18, 19].

The ability of human DCs to acquire and crosspresent antigens from dying cells was first studied using influenza-infected monocytes [20]. In these studies, monocyte-derived DCs could crosspresent viral antigens derived from apoptotic, but not necrotic cells. Monocytes or macrophages were unable to mediate crosspresentation of viral antigens under these conditions. Subsequent studies have shown that human monocyte and $CD34^+$ progenitor-derived DCs, loaded with irradiated tumor cells, can elicit tumor specific T-cell responses *in vitro* [14, 16, 17, 21, 22]. Other studies have suggested that DCs are capable of presenting antigens from necrotic cell lysates of tumors or virally infected cells as well [23–25]. Injection of tumor-loaded DCs can induce tumor-specific immunity and mediate protection in tumor challenge experiments in mice [24, 26]. The uptake of apoptotic tumor cells by DCs is linked to the expression of several receptors such as $\alpha v\beta 3$, CD36 and receptors for phosphatidylserine expressed on the surface of apoptotic cells [27]. It is not clear at present as to which of these pathways is critical and whether the specific receptors involved in the uptake of dying cells impacts the fate of the antigen. Uptake via $\alpha v\beta 5$ has been shown to recruit a CrkII-Dock180 molecular complex, which in turn activates rac1 and cytoskeletal machinery [28]. Specific (or preferential) targeting of apoptotic cells or derived particulate antigens to distinct receptors on DCs (or their blockade) may potentially impact the efficiency or outcome of crosspresentation.

54.4
Uptake of Immune Complexes and Opsonized Pathogens and Tumor Cells

Fcγ receptors on DCs can help mediate efficient uptake of immune complexes, and opsonized cells [29, 30]. Moreover, opsonization of the apoptotic cells by immature DCs can be enhanced by complement factor iC3b [31]. In prior studies, we had shown that coating myeloma tumor cells with anti-syndecan-1 antibody greatly enhances the crosspresentation of tumor-derived antigens via DCs [19]. Similar findings have been reported using immune complexes of tumor-associated anti-

gens [32]. FcγR-mediated crosspresentation does not simply depend on the presence of Fcγ receptor on DCs, as Langerhans cells (LC) which also express Fcγ receptors do not seem to be able to crosspresent immune-complexed antigen [33].

Biologic effects of signaling via the Fcγ receptor are determined by the balance between activating and inhibitory receptors, both of which are generally co-expressed and co-engaged by immune complexes [34]. Recently it was demonstrated that targeting of antigen to Fcγ receptors on DCs leads to Th1 $CD4^+$ and $CD8^+$ effector responses *in vivo* [35]. Tumor immunity specific for the ovalbumin expressing tumors was provided by immunization with wild type, but not FcγR-deficient DCs loaded with ovalbumin containing IC. Interference with the inhibitory signal delivered by FcγRIIB on DCs (using FcγRIIB KO mice) converted ICs to potent DC maturation agents and resulted in effective T-cell activation. Applying this approach to immunization with DCs pulsed *ex vivo* with ICs, antigen-specific $CD8^+$ T cells were generated *in vivo* achieving efficient protective immunity in a murine melanoma model [36]. These data imply that ICs normally serve to maintain tolerance through the binding to inhibitory FcγRs on DCs, but they can be converted to potent immunogenic stimuli by selective engagement of activating FcγRs. Availability of a new monoclonal antibody allowed the feasibility of selective blockade of the inhibitory Fcγ receptor on human DCs. Using this antibody, we have recently shown that blockade of inhibitory Fcγ receptor leads to DC maturation and enhanced immunity to antibody-coated tumor cells [37]. Monoclonal antibodies have emerged as an important new tool in the therapeutic armamentarium against cancer. Selective modulation of the Fcγ receptor system should be an attractive approach to optimize loading of tumor antigens to DCs both *ex vivo* and *in vivo*, using antitumor monoclonal antibodies already in the clinic.

54.5 Uptake of Heat Shock Protein–Peptide Complexes

Heat shock proteins (hsps) are highly conserved molecules that serve to chaperone proteins between subcellular compartments [38]. Hsps are released by necrotic cells and can serve to mature DCs and enhance their function [39]. APCs internalize hsps with bound peptides via receptor mediated endocytosis, resulting in antigen presentation via MHC class I molecules [40, 41]. At least two receptors on DCs, CD91 and LOX-1 have been implicated in the uptake of hsp–peptide complexes for crosspresentation [42, 43]. However, much remains to be learnt about the mechanism of hsp-mediated crosspresentation, and its role in tumor immunity has been debated [44]. Nonetheless, targeting hsps remains an attractive way of enhancing the efficacy of tumor vaccines.

54.6 Exosomes as Sources of Multiple Tumor Antigens

Exosomes are multivesicular bodies derived from hematopoietic and tumor cells [45]. Exosomes originate from multivesicular late endosomes, and are secreted upon fusion of these bodies with the plasma membranes. Exosomes are enriched in MHC I peptide complexes, and heat shock proteins that could account for their immunogenicity [46]. However in spite of high hsp content, exosomes derived from DCs or tumors do not seem to induce DC maturation and are not immunogenic, unless they are delivered with additional stimuli. Exosomes have been isolated from patients with advanced tumors and shown to be a source of defined tumor antigens such as Her-2/Neu [47]. Clinical studies using exosomes to target tumors have now begun. Tumor-derived exosomes can serve as a source of antigen for crosspresentation by DCs, and DCs loaded with such exosomes induce tumor rejection. Surprisingly, the effect is not entirely tumor specific, as exosomes from some tumors can protect against allogeneic tumors, while the protection using irradiated tumors is largely tumor specific [48].

54.7 Role of C-type Lectin Receptors

The term C-type lectin receptor (CLR) defines carbohydrate binding molecules that bind ligands in a calcium dependent manner [49]. DCs express several CLRs that mediate distinct patterns of binding to both exogenous and endogenous ligands [50]. Antigens bound to at least some of the CLRs can be efficiently targeted to late endosomal/lysosomal compartments for degradation and presentation to T cells [51]. However several other factors including the nature of the maturation stimulus probably determine the immunologic outcome of the uptake. Due to the efficiency of antigen targeting, particularly for presentation to $CD4^+$ T cells, targeting CLRs *in vivo* may have major implications on vaccine design [49]. However, improved understanding of the fate and regulation of presentation of CLR targeted antigens as well as the pathogenic patterns recognized by these receptors is needed. It is possible for example, that recognition of different molecular patterns by CLRs leads to differential processing of phagocytosed antigen, and different outcomes for antigen presentation, including induction of tolerance [52]. The latter may be an important aspect of antigen targeting via CLRs.

54.8 Other Routes of Antigen Entry for Crosspresentation

In addition to particulate antigens, DCs can also acquire soluble antigens by pinocytosis and crosspresent them on MHC I, albeit with much reduced efficiency as compared to particulate antigens [53, 54]. Recent studies by Cresswell's group have

shown that this occurs due to the access of pinocytosed antigen to the perinuclear endoplasmic reticulum in DCs [55]. Although this pathway is likely to be quite inefficient for improving vaccination, it may provide an approach for maintaining tolerance to soluble antigens. Yet another pathway for access of antigens to DCs is via gap junctions between DCs and surrounding cells. This was recently demonstrated as a mechanism for antigen transfer between LC and keratinocytes [56]. The importance of this gap junction mediated immunologic coupling to crosspresentation *in vivo* remains to be explored.

54.9
Processing of the Antigenic Cargo

Recent studies have provided key insights into the fate and processing of phagocytosed antigen prior to loading to MHC I for crosspresentation. Three groups recently reported the existence of a process whereby phagosomes fuse with vesicles derived from the endoplasmic reticulum (ER) to create a hybrid compartment [57–59]. The resulting hybrid compartment contains MHC I molecules, along with other components required for MHC I loading, such as transporter associated with antigen processing (TAP), tapasin, calreticulin and ERp57. Phagocytosed antigens are then briefly transported to the cytosol by an undefined mechanism that may involve the Sec61 complex, a multimolecular channel normally used to transport secreted proteins into the ER. In the cytosol, the proteins are degraded by the proteasome, before transport back into the phagosome via the TAP complex for MHC I loading. Although the reasons behind the need for involvement of the ER are not intuitively obvious, these data provide several new molecular targets that may be exploited to manipulate crosspresentation in vaccine design. One important new insight is that the crosspresented antigen derived from the donor cell is a proteasomal substrate and not an end product, and needs to be processed in the antigen presenting DCs [60, 61]. Inhibition of proteasomal function in the donor cell may therefore enhance the efficiency of crosspresentation, and has major implications for designing approaches for antigen loading of DCs for vaccination.

54.10
Nature of the Antigenic Cargo

There is increasing evidence that the nature of the cargo has an influence on the processing and presentation of exogenous antigen in the phagosome [3]. This depends both on the nature of the antigenic material, and the associated chaperone molecules such as hsps or immune complexes. In some instances, as with hsps or immune complexes, this “chaperone effect” is largely influenced by the biology of the uptake receptor, as discussed earlier. However the nature of the cargo in the phagosomes may also be monitored based on the presence of pathogen associated molecular patterns (PAMPs). For example, TLR ligands in the phagocytic material,

that stimulate endosomal TLRs can lead to the maturation of the phagosome, allowing crosspresentation of the antigenic contents [62]. This may represent a mechanism by which DCs can differentially process apoptotic bodies derived from normal versus infected cells. For example, ligation of Toll receptor 3 (TLR3) in $CD8^+$ DCs by double stranded RNA in virally infected cells, may be critical for crosspresentation of antigens derived from these cells [63]. Signaling via Toll receptors may be critical for efficient cross priming *in vivo* and needs to be exploited for clinical studies [64].

The processing and presentation of antigens from dying tumor cells may also depend on the mode of induction of cell death. Most of the clinical studies of DC immunotherapy to date have utilized lysates derived from tumor cells, or irradiated tumor cells (to induce apoptosis) [65]. However other modes of induction of apoptosis such as cancer chemotherapeutic agents, or biologic methods such as natural killer cells or cell death inducing ligands may provide different substrates for DCs, which may influence the presentation of tumor antigens. For example, alkylating agents commonly used as chemotherapies work by intercalating into DNA and inducing DNA damage. However these changes in the DNA may well alter their immunogenicity and impact DC maturation/antigen presentation by pattern recognition [66]. For example, Toll-like receptor ligation by autologous DNA sequences may be critical in the pathogenesis of lupus [67]. Another important unknown is whether the involvement of different initiator caspases for the induction of apoptosis by different methods to induce apoptosis leads to different immune responses to dying cells *in vivo*. Improved understanding of these processes will facilitate optimization of loading of dying tumor cells to DCs for vaccination.

54.11
Regulation of Crosspresentation during Dendritic Cell Maturation

Similar to MHC II presentation, MHC I crosspresentation may be developmentally regulated in DCs. Signals mediated by Fcγ receptors, inflammatory compounds, $CD4^+$ T cells and disruption of intercellular contacts have been reported to induce crosspresentation [68–71]. It is important to distinguish here between the enhancement of formation of MHC–peptide complexes and increased T-cell stimulation. The latter depends on increased T-cell stimulatory capacity of DCs, a process termed DC maturation or activation, that is reviewed elsewhere in this book. A report by the Amigorena group has shown that mature DCs downregulate uptake and delivery of antigen to the crosspresentation pathway [69]. This downregulation may allow mature DCs to focus their crosspresenting activity on antigens captured at the time of activation. These data also suggest immature DCs as targets of antigen loading for crosspresentation. Mature DCs concentrate endogenous ubiquitinated proteins and defective ribosomal entry products in specialized cytosolic aggregates called dendritic aggresome-like structures (DALIS) [72]. Formation of DALIS may allow the DC proteasomal machinery to focus more on exogenously acquired antigen. It is also possible that exogenously acquired antigen gains access

to such storage compartments, in order to generate a mechanism for antigenic memory of crosspresented antigen. Our current understanding of the quantitative aspects of crosspresentation, compared to endogenous presentation are rather sketchy [73]. However greater understanding of the quantitative aspects of processing and storage of the crosspresenting antigen will have important implications for vaccine design.

54.12 Role of Dendritic Cell Subsets in Crosspresentation

DCs are heterogeneous and consist of several subsets with distinct biology. In mice, $CD8^+$ subset of DCs has been implicated as the major subset involved in crosspresentation of cellular antigens [74–76]. This is not directly related to the phagocytic ability, as other DC subsets capable of antigen uptake seem to be weak at crosspresentation [77]. One possibility is that the dermal/interstitial DCs are involved in transport of cellular antigen and presentation to $CD4^+$ T cells, but this subset is unable to crosspresent tissue derived antigen to $CD8^+$ T cells. Crosspresentation of antigens taken up by these DCs may therefore require transfer of antigen to $CD8^+$ DCs in the lymph node, which then crosspresent this antigen [78]. The human counterpart of murine $CD8^+$ DCs is not known. Most studies of crosspresentation by DCs have been based on *in vitro* studies using monocyte derived DCs. Culture of $CD34^+$ hematopoietic progenitors can lead to differentiation of DCs resembling a mixture of LCs and interstitial DCs. Although LCs are less phagocytic than monocyte derived DCs, recent studies have shown that they are also capable of crosspresentation [79]. In some studies, this required treatment of LCs with interferon-γ [80]. In contrast to myeloid DCs, plasmacytoid DCs appear to be poor at phagocytosis and cross priming. However, interferon-α secreted by these cells can lead to activation of other DCs and enhanced crosspresentation [81]. Understanding the role of different DC subsets in crosspresentation *in vivo* has obvious implications for optimizing antigen delivery. This is particularly relevant for *in vivo* targeting of DCs, such as with DNA vaccines.

54.13 Some Approaches to Improve Antigen Loading of Dendritic Cells for Clinical Vaccination

The ability of DCs to crosspresent exogenous antigens on MHC I provides an opportunity to exploit these properties to load antigens from tumors or viruses for DC based immunotherapy [8, 65]. Most of the early studies targeting this property have been in the setting of therapeutic tumor vaccines, and utilized lysates [9, 82–85] or apoptotic bodies derived from tumor cells [86, 87]. These data demonstrate that this approach is feasible and well tolerated. Importantly, development of clinically limiting autoimmunity has not been observed. These studies have also demonstrated

the ability of these cells to enhance T-cell immunity *in vivo*. However the overall clinical and immunologic results with these approaches have been modest and emphasize the need to improve the antigen loading and immunogenicity of DCs.

54.14 Concluding Remarks

As discussed above, newer insights into DC biology suggest several variables to improve DC vaccination (Table 54.1). A critical aspect is the importance of receptor targeting for optimal uptake and processing of antigen. For example, immune complexed antigens taken up by FcγR can be presented up to 100-fold more efficiently than soluble antigens ingested by pinocytosis [88]. Opsonization of tumor cells by antitumor monoclonal antibodies leads to enhanced crosspresentation of tumor antigens by an FcγR dependent mechanism [19]. Antigen uptake and crosspresentation machinery are more active in immature DCs, which are rational targets for antigen loading. The ability of DCs to stimulate T cells against exogenous antigens is strongly linked to their maturation status. However the nature of the maturation stimulus also matters. In particular, ligation of Toll receptors, stimuli via CD40 ligand, and induction of interferon-α/β signaling may be critical for efficient crosspresentation. Another important variable may be the subsets of DCs responsible for crosspresentation *in vivo*. Although this is better understood in mice, improved understanding of crosspresenting DCs *in vivo* in humans is needed to optimize antigen targeting and improve vaccination against cancer and pathogens.

Tab. 54.1 Some variables to improve crosspresentation of tumor-derived antigens via dendritic cells.

Nature of the antigen	Particulate antigen
	Engagement of intracellular TLRs or pathogen recognition receptors
	Exosomes
Targeting the uptake receptors	Receptors for uptake of dying cells.
	Fcγ receptors (opsonized tumors, immune complexes)
	Receptors for heat shock proteins
Maturation stimuli	Toll receptors
	CD40/40L interactions
	Type I interferons
	Modulating Fcγ receptors
	Innate lymphocytes
DC subsets	$CD8^+$ Dendritic Cells (in mice)
	Plasmacytoid Dendritic Cells
	Langerhans cells

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55
Nucleic Acid Transfer

Niels Schaft, Jan Dörrie and Dirk M. Nettelbeck

55.1
General Introduction

Therapeutic vaccination as a treatment for serious diseases like cancer or virus infections is a promising approach which has two major characteristics that make it very attractive: (1) specificity and (2) establishment of immunologic memory, which may provide long-term protection against recurrence of disease. Being the most efficient antigen-presenting cells (APCs), dendritic cells (DC) are the best adjuvant to trigger an effective immune response that the organism fails to establish without help by vaccination, as in the case of cancer or AIDS. In order to make these vaccinations successful, efficient strategies to introduce relevant antigen (Ag) into these DC are needed. Therefore, many groups in the field of DC research concentrate on developing and optimizing such strategies, with the aim of using Ag-loaded DC to stimulate MHC class I- and class II-restricted antitumor or antiviral immunity (Fig. 55.1).

Tumor- or viral-Ag can be delivered to DC in many different ways. The easiest and most direct approach is exogenous Ag-loading of DC with previously characterized (i.e. amino acid sequence, HLA-restriction) and synthetically produced antigenic peptides (Fig. 55.1) originating from viral proteins [1–5], or from proteins derived from tumors, such as melanoma [6–10]. This method proved to be a very efficient loading strategy, resulting in tumor-specific cytotoxic T lymphocyte (CTL) and T helper cell 1 (Th1) responses [6, 9, 11–13]. However, this method is limited to known viral and tumor epitopes for the synthesis, and in addition limited by the patient's HLA-restriction pattern [13, 14]. As a result, many patients will not be eligible for peptide-loaded DC vaccination. A possible solution is the use of isolated native peptides for DC peptide-loading [15–17]. However, this is highly dependent on the amount of tumor tissue available, as is the alternative approach of loading the DC with tumor lysate. The latter method of Ag-delivery takes advantage of the DC biology itself, e.g. the capability of the DC to phagocytose tumor lysate or other sources of Ag, resulting in epitope presentation by MHC class I and II molecules (as described in more detail in Chapter 54) (Fig. 55.1).

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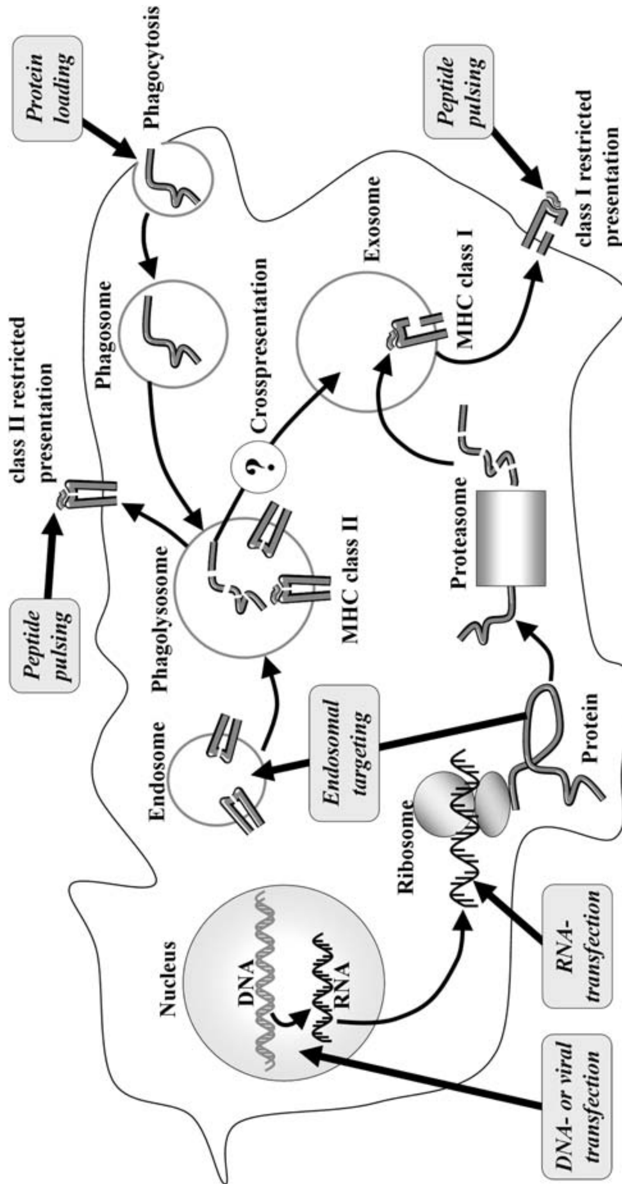

Fig. 55.1 Manipulating Ag presentation of DC. The cellular pathways that lead to MHC class I- and II-restricted presentation of Ag can be manipulated at several key points. The natural abilities of the DC are utilized in protein loading: DC efficiently phagocytose extracellular material. Included proteins are degraded to peptides, which bind to MHC class II in intracellular vesicles and are then transported to the cell surface where they are presented. A small portion of Ag can also gain access to the MHC class I presentation pathway by a mechanism termed crosspresentation. However, the exact molecular principles for this remain unknown. A more efficient path to MHC class I presentation is transfection. DNA- or viral transfection introduce exogenous DNA into the DC's nucleus, where it is transcribed like genomic DNA. Encoded genes are then expressed, degraded and presented via MHC class I. RNA-transfection cuts short the step of transcription and can be directly translated into proteins, which are then presented in a similar way. By genetically fusing the expressed Ag to appropriate signal peptides, endosomal targeting of the proteins is possible, resulting in class II presentation. Both MHC class I and II are also accessible for peptide pulsing if the MHC haplotypes are adequate and suitable peptides are available.

An elegant way to gain direct access to the MHC class I and II processing pathways is the expression of Ag as full-length protein within the DC. This allows the generation and presentation of all biologically relevant peptides that can be derived from the protein of interest, since the DC's own protein degradation and presentation machinery is used. Mainly two methods are used in this approach: (i) viral vectors containing the coding sequence of the Ag of interest, and (ii) nonviral transfection methods with DNA or RNA also coding for the Ag of interest (Fig. 55.1).

Transfer of nucleic acids into DC has several advantages compared with peptide-loading of DC: (i) the expression of the whole polypeptide-chain allows the cells' degradation machinery to potentially generate all possible MHC class I- and class II-presented peptides (e.g. by including protein trafficking signals) – known or unknown – and is therefore not limited to certain HLA-haplotypes; (ii) simultaneous presentation of multiple epitopes derived from the same Ag is possible; (iii) the whole target cell transcriptome (e.g. whole tumor RNA) can be expressed in DC; (iv) DNA/RNA provide the DC with an internal source of peptides for further MHC-peptide loading, which is absent when DC are loaded with peptide resulting in a gradually loss of presented epitopes on the DC membrane; (v) DNA/RNA can be produced in large quantities at low costs; and (vi) the activity of the DC can be modulated by the gene transfer vector or immunostimulatory factors encoded by the DNA/RNA.

In the following sections, we will describe viral and nonviral Ag delivery to DC in more detail, with emphasis on Ag delivery by adenoviral vectors and transfection of nucleic acids, two techniques used and optimized in our and other laboratories.

55.2 Antigen Delivery to DC by Adenoviral Gene Transfer

Transfer of DNA into DC provides a promising tool for prolonged expression and, following intracellular protein processing, presentation of antigens (Fig. 55.1). Besides applications in *ex vivo* DC vaccination protocols, *in vivo* gene transfer into DC represents an attractive concept for DC-mediated vaccination – or tolerization – without the need for purification and *ex vivo* handling of DC. In this regard, an appealing feature of DNA vaccination/tolerization, besides Ag delivery, is the opportunity to manipulate DC biology and the immune response by transfer of genes that encode, for example, migration-promoting, anti-apoptotic, or immunomodulatory factors. The key challenge, however, is to introduce DNA efficiently into DC – specifically into the DC's nucleus. Over and above *specific* DNA transfer into DC, maybe even specific Ag expression in *mature* DC, might be required for *in vivo* protocols. Simply adding recombinant DNA to DC does not do the trick. Thus, a gene transfer vector is required.

Viruses are nature's DNA transfer professionals which feature highly developed equipment to introduce their genomes into the nuclei of eukaryotic cells. Clearly, adenoviruses have been preferred gene transfer vectors in pre-clinical and clinical gene therapy [18], especially if transient but strong transgene expression is desired as for genetic vaccination. Key advantages are their effective gene transfer machinery, their high structural and genomic stability, their nonintegrating genome, low pathogenicity, and the ease of adenovirus handling and of their production at high titers [18]. Adenoviruses have long been an important tool in molecular biology research. They have been essential for the unraveling of fundamental biological processes such as DNA replication, gene transcription and splicing. Consequently, adenovirus structure, genome and life cycle have been uniquely well characterized,

thus establishing a key advantage for molecular vector modifications as required for gene therapy. Examples for such modifications are the incorporation of therapeutic genes into the virus genome and strategies for the targeting of gene transfer to choice cell types (see below). Hence, adenoviruses feature the fundamental requirements for genetic DC vaccination: gene transfer efficiency and the potential for targeting of gene transfer/expression to DC.

Research into adenoviral vaccines was inspired by early gene therapy studies, which examined adenoviral gene transfer in mice. This work revealed that adenoviral gene transfer induces potent innate and adaptive immune responses to transgene-encoded proteins (for example [19]). While these observations established a major hurdle for adenoviral vector-mediated gene correction, they led to investigation of adenoviruses as vaccine carriers.

55.2.1 Recombinant Adenovirus as Gene Transfer Vector

Adenovirus virions consist of a double stranded DNA genome, which is packed into a protein capsid of approx. 100 nm in diameter (Fig. 55.2A). The latter is an icosahedron with surfaces built of hexon protein and vertices made of penton base and the cell-binding antennae-like fiber protein. To date, more than 50 different human adenovirus serotypes have been described. Adenovirus gene transfer vectors are derived from the particularly well characterized adenovirus serotype 5 (Ad5). Infection of Ad5 and most other serotypes initiates with virus binding to host cells via interaction of the virus fiber knob domain with the cellular coxsackie and adenovirus receptor (CAR, Fig. 55.3A). Virus internalization by receptor-mediated endocytosis is triggered by interaction of the penton base with cellular integrins. After endosomal disruption and migration of virions to the nuclear membrane, the virus genome is transferred into the nucleus. The adenovirus genome is densely packed with viral genes. It is flanked by inverted terminal repeats (ITRs), which are essential for DNA replication (Fig. 55.2B). Early genes (E1 to E4 gene regions) are expressed before replication of the virus genome, late genes (L1 to L5), which encode mostly structural capsid proteins, are expressed subsequently. After assembly of virions from structural proteins and genomes, a new virus generation is released from the host cell (for a more detailed review see [20]).

Adenovirus gene transfer vectors are engineered by replacing essential viral genes with the transgene(s) of interest (Fig. 55.2B). Thereby, the vectors feature cell entry and nuclear transfer of the recombinant genome similar to wild-type virus. However, virus gene expression and genome replication are blocked, while the transgene is expressed from a transcription control element (promoter) inserted in such *transduced* cells. In first generation vectors, viral E1 and E3 genes are deleted. E1 proteins are key regulators of gene expression and replication, whereas E3 proteins are dispensable for virus replication but have immunosuppressive functions. In consequence, adenoviral gene transfer vectors lack the viral immunosuppressive activities – an important feature for the application of these vectors in immunotherapy. After establishing these so-called first generation vectors, gene therapists

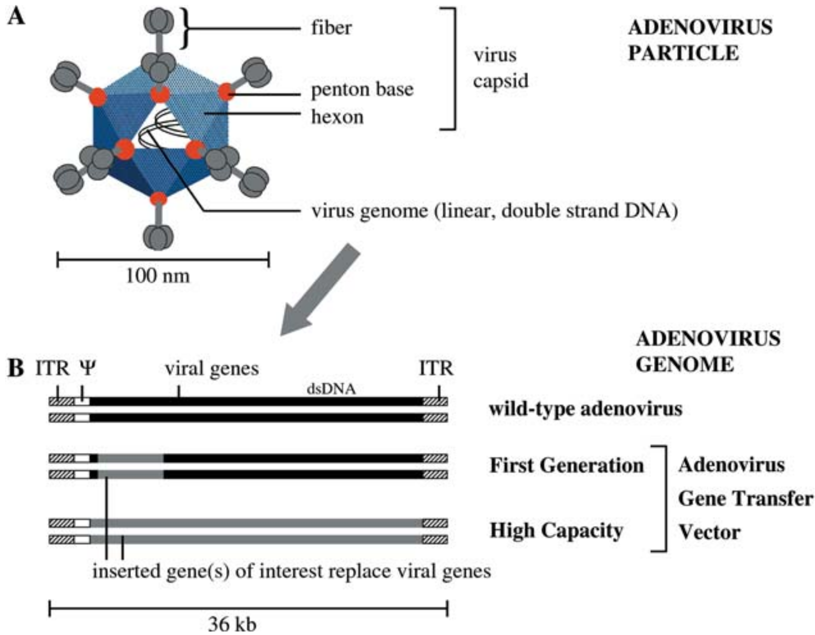

Fig. 55.2 Capsid (A) and genome (B) structure of adenoviruses and adenovirus vectors. The adenovirus capsid is an icosahedron that consists of 20 triangular surfaces and 12 vertices. It is built of 240 hexon proteins, 12 penton bases, 12 fibers and minor capsid proteins. The virus genome is a double strand DNA of approx. 36 000 base pairs with inverted terminal repeats (ITRs), a packaging signal (ψ) and the virus genes. The ITRs are required for replication of the virus genome, and ψ mediates incorporation of replicated genomes into pre-formed capsids. In first generation adenoviral gene transfer vectors the essential viral E1 genes (and E3 genes) are replaced by the transgene of interest. Hence, these vectors are replication-deficient and are produced in E1-transcomplementing cell lines. All viral genes are deleted in high capacity adenovirus vectors, but they retain ITRs and ψ. High capacity vectors are produced by co-infection with helper adenoviruses, which complement the viral gene products and need to be separated from the high capacity vectors after production.

have achieved complete removal of viral genes, just retaining the virus ITRs. Such vectors have been named high capacity adenovirus vectors (Fig. 55.2B), because they allow the insertion of heterologous DNA of up to 36 kb. Thus, multiple genes can be incorporated into these vectors, which is important if immunomodulation in addition to (multiple) Ag delivery is considered (see below). However, the production of these vectors by amplification in eukaryotic producer cells requires co-infection of "helper viruses" that complement all viral genes. Helper viruses need to be separated from the vectors after virus production. In contrast, first generation adenovirus vectors can be produced in cells that stably express the few viral genes that are required to complement vector replication (for a more detailed review see [18]).

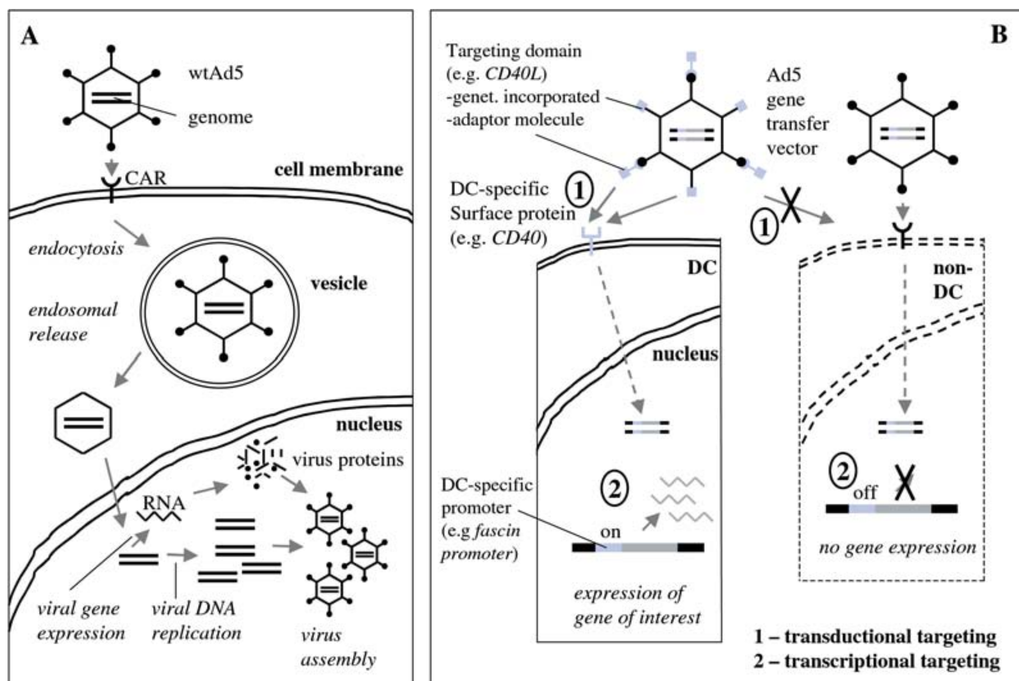

Fig. 55.3 Adenovirus infection cycle (A) and targeting of adenoviral gene transfer vectors (B). Adenoviruses bind to their cellular receptors (in case of serotype 5 the coxsackie and adenovirus receptor, CAR) with the knob domain of the fiber. Receptor-mediated virus internalization is triggered for most serotypes by binding of the penton base to cellular integrins. After endosomal release and trafficking to the nucleus, the viral genome is transferred into the nucleus. Expression of early genes (E1 to E4) is followed by replication of the viral genome and expression of late genes (L1 to 5) follows. Finally, the virus capsid is assembled, the genome is packaged into the capsids and ultimately the progeny viruses are released by cell lysis. Targeting of adenovirus vectors has been achieved at the cell entry (transductional targeting, 1) or at the gene expression level (transcriptional targeting, 2). For the former, cell binding ligands are either genetically incorporated into the capsid or are physically attached to the capsid in form of an adaptor molecule. The latter is achieved by a tissue-specific transcriptional control element (promoter) that drives transgene expression.

Targeting of gene transfer, which is enabling gene expression in a specific cell type or at a specific time, is a major goal for most gene therapy strategies and might also be critical for genetic vaccination with adenoviral vectors. Targeting has been achieved mainly by two strategies (Fig. 55.3B). *Transductional targeting* is based on binding of the gene transfer vector via a targeting domain to a cell surface molecule that is specifically expressed on the targeted cell, thereby triggering specific entry into these cells [21]. In contrast, *transcriptional targeting* by means of a cell type specific or inducible regulatory DNA sequence (promoter) results in specific gene expression, even after nonspecific cell entry of the vector [22]. Vectorologists have benefited from advanced molecular virology and recombinant DNA technology to implement both strategies for adenovirus vectors.

55.2.2
Adenoviral Gene Transfer into Dendritic Cells in Vitro

For effective vaccination, DNA transfer to DC is required to be efficient, nontoxic and nonperturbing for the fragile DC phenotype. Although DC turned out to be more resistant to adenoviral infection than other cells, >90% transduction efficacy is usually achieved at high titers (see for example [23]). Note that modification of virus tropism successfully augmented adenoviral transduction of DC (see below). Most studies on adenoviral gene transfer to DC have investigated first generation Ad vectors. Recent studies, however, showed that in direct comparison helper-dependent Ad resulted in superior transgene expression *in vitro* and also in a stronger CTL activation in an *ex vivo* setting [24].

In mostly direct comparison to physical methods of DNA transfer, such as liposomes, electroporation, and biolistic methods, adenoviral vectors showed superior gene transfer efficiency and gene expression that persisted for more than one week [23, 25–29]. Importantly, physical methods were reported to be substantially toxic to DC, whereas high titers of adenoviruses that resulted in >90% transduction of DC were tolerated. Note that transduction efficacy and toxicity is considerably different for RNA transfer (see [30] and Section 55.3)

Other viral vectors used for genetic DC-mediated vaccination were adopted from the field of gene therapy, such as *Vaccinia* virus-based vectors (DNA virus; [31–37]), *Herpes simplex* virus vectors (*HSV-1*, DNA; [38]), *Influenza* virus vectors (RNA), adeno-associated virus vectors (DNA; [39, 40]) lentivirus (RNA; [41–47]) or retrovirus (RNA; [48–50]). However, not all of these are suitable for transduction of DC because they cannot transduce nonproliferating cells (retrovirus), show cytopathic effects (*Vaccinia* virus, *HSV-1*) or block induction/induce down regulation of maturation markers and T-cell stimulatory capacity of DC (*Vaccinia* virus, *HSV-1*; [51]).

How does transduction with adenovirus influence DC biology? The vast majority of publications shows that adenovirus transduction is not toxic for DC and does not block DC maturation by external stimuli at titers that result in high gene transfer efficacy. In this regard, it is important to note that the E3 gene region, which encodes viral proteins that interfere with the host anti-adenovirus cellular immune response, is deleted from the genome of adenovirus vectors. There is no consensus on the question if adenovirus transduction itself does induce DC maturation. Several groups report that adenovirus transduction *per se* does not induce maturation and T-cell stimulatory activity of murine or human DC (for example [23, 26, 52, 53]). Others show increased expression of at least some maturation markers and augmented T-cell activation by DC after adenovirus gene transfer (for example [54–60]. Furthermore, a synergism of adenovirus infection and other maturation stimuli, or of antiviral immune induction with antitumoral responses have been described [55, 60]. The reason for such divergent reports is the fragile phenotype of DC, which is easily skewed by a multitude of factors, among them the method and quality of DC generation, handling and purification technique, presence of serum or other factors in the culture media, purity and titer of adenovirus, as well as the organ, donor, species and strain from which DC originate [58, 61]. These consider-

ations underline the importance to characterize the maturation status of transduced DC for each protocol individually. Detailed *in vivo* analysis of potential DC activation by adenoviral vectors is still awaited.

Tropism-modification of Ad5-derived vectors is critical to achieve efficient gene transfer into DC, because DC lack expression of the adenovirus receptor CAR [52, 53, 55]. Moreover, by incorporation of DC-specific ligands into the virus capsid, tropism-modification, can be a means to achieve not only enhanced, but also specific gene transfer into DC. This strategy, named transductional targeting, might open an avenue for *in vivo* vaccination with adenoviral vectors. Targeting ligands can be a tool not only for efficient or targeted gene transfer, but also for ligand-induced DC maturation, thereby establishing a very interesting link between DC gene transfer and maturation. An up to 100-fold augmentation of Ad gene transfer into DC has been demonstrated by fiber chimerism, that is by replacing the Ad5 cell-binding fiber domain with the corresponding domain of a different adenovirus serotype, such as serotypes 3 (10-fold increase [62]), 11 (6-fold increase [63]) or 35 (10- to 100-fold increase [64, 65]). These adenovirus serotypes bind to receptors different from CAR. CD46 has been identified as a cellular receptor for adenovirus serotypes 11 and 35 [66, 67]. CD80 and CD86 were reported to mediated cell entry of adenovirus serotype 3 [62]. Similar results were achieved by incorporating an integrin-binding RGD peptide into the adenovirus capsid [68–70]. Curiel and co-workers succeeded in combining enhanced and DC-selective gene transfer with the induction of DC maturation by targeting adenovirus vectors to CD40. This was demonstrated by means of bispecific adapters which link a CD40-binding antibody to the Ad5 capsid [52, 53, 71–73], or by incorporating CD40L into a genetically engineered virus capsid [74]. CD40-targeted Ad gene transfer of E7 protected mice from HPV-16 induced tumors [53]. In human skin explants, this strategy resulted in targeted and enhanced gene transfer to DC, resulting in an augmented and prolonged CTL-stimulating capacity [75].

Transcriptional targeting can mediate DC-specific gene expression or even maturation-specific gene expression. The latter might be critical in vaccination settings where tolerization, that has been reported to be induced via Ag presentation by immature or semi-mature DC [76], needs to be avoided. Transcriptional targeting of GFP or β-Gal marker gene expression after plasmid transfection has been demonstrated for the fascin promoter in mouse and human DC [77–79]. Notably, compared with nonspecific promoters, expression of antigens from the fascin promoter resulted in a qualitatively different immune response with similar induction of CTLs, but a weaker antibody response. Further DC-specific promoters that have been characterized are those of Dectin-2, which showed activity in Langerhans cells [80, 81], and the promoter of the maturation marker CD83 [82]. However, it remains to be determined whether DC-specific promoters retain their activity and specificity profile in adenovirus vectors, because promoter activity and specificity might be influenced by viral regulatory elements or viral proteins.

55.2.3
Adenoviral Antigen Delivery to DC for Ex Vivo Tumor Vaccination in Mouse Tumor Models

Dendritic cells loaded with tumor antigens by means of adenoviral gene transfer have been examined for feasibility as protective or therapeutic tumor vaccines in mice. Several studies demonstrated tumor cell killing, tumor protection, or reduced tumor growth, respectively, exploiting model antigens, such as OVA, β-Gal, or polyoma middle T [68, 83–86], tumor virus antigens, such as HPV16 E7 or EBV LMP2A [53, 87], or tumor-associated antigens (TAAs), such as melanoma differentiation antigens [54], AFP and Her-2/Neu [88] (see [89] for a more complete list). Furthermore, these studies offered insight into the nature of anticancer immune responses in mice and revealed potential obstacles resulting from the application of adenoviral vectors.

After initial gene therapy studies in mice had reported the induction of strong immune responses to various transgene-encoded proteins after adenoviral gene transfer, the potency of adenoviral delivery of pathogen-derived antigens was investigated next. Studies involving various pathogens, including HIV, rabies virus, CMV, EBV, and SARS (for an overview see [90]) demonstrated the induction of long-lived protective humoral (mainly of the IgG2a isotype) and $CD8^+$ biased cellular immune responses to the transgene product after both systemic and mucosal vaccination. Fast immune activation after single virus injection, as observed by various groups, is considered advantageous for post-exposure applications aiming at the control of outbreaks. However, adenoviral vaccines also induced anti-adenoviral humoral immunity that limited the efficacy of repeated vaccination.

Tumor vaccination studies suggest that, at least for untargeted Ad5 vectors, *ex vivo* Ad transduced DC vaccines are superior to *in vivo* administration of the same vectors and altered the character of immune responses in favor of strong cellular immunity [54, 83, 85, 91–94]. Adenovirus transduced DC were superior to peptide-loaded DC or to DC loaded with tumor lysate in multiple vaccination studies [83, 95, 96]. Importantly, previous exposure to adenovirus and neutralizing anti-adenovirus antibodies did not abolish TAA-specific immune responses induced by adenovirus transduced DC [53, 54, 83, 93, 94, 97]. Moreover, mice immunized with Ad transduced DC mounted no significant antiviral CTL responses [54, 83, 85]. These results suggest that both Ad/DC vaccination of individuals seropositive for neutralizing anti-Ad antibodies and repeated Ad/DC vaccinations are feasible. However, it remains unclear whether rodents are suitable models for the prediction of antiviral immunity in patients. Notably, in most studies with rodents one vaccination with Ad/DC was sufficient to induce effective and prolonged anticancer immunization.

More recent *in vivo* studies in mice provided insight into cellular and molecular mechanisms of antitumor immune activation after vaccination with Ad-transduced DC. Experiments with mismatched MHC molecules found that allogeneic DC were not able to induce specific immunity, whereas vaccination with syngeneic DC was successful. These experiments indicated that T cells are directly activat-

ed by *ex vivo* transduced DC and not by cross presentation [97, 98]. This conclusion was confirmed by the observation that transduced MHC Class II knock-out DC were unable to protect mice from tumor challenge [98]. This study also suggests that $CD4^+$ T cells are involved in the induction of antitumor immunity as supported by several studies in knock-out mice and in T-cell depletion assays [29, 53, 54, 70, 83, 88, 94, 99–101]. There are conflicting reports, however, on the requirement of $CD4^+$ T cells in the effector phase of the antitumor immune response. Several investigators observed by immune depletion before tumor challenge that $CD4^+$ T cells are not required [102, 103]. Others found that $CD4^+$ cells are involved in the effector phase of antitumor immunity [92, 101] or even observed by immune depletion or in knock-out mice that antitumor immunity was independent of $CD8^+$ T cells [85, 93, 98]. In the latter study, immunity was completely or partially abrogated by depletion of $CD4^+$ T cells or NK cells, respectively.

Adenoviral vectors, in addition to implementing a means for Ag loading of DC, represent also an opportunity to modify the character of immune responses by gene transfer of immune regulatory factors. Such strategies aim to derive enhanced DC after transfer of genes that induce DC maturation, enhance DC motility, improve the stimulatory capacity of DC – for example by expression of co-stimulatory factors or secretion of proteins – or prolong DC lifespan by expression of anti-apoptotic factors. DC genetically modified to express IL-12 resulted in tumor growth arrest and regression [103–108]. In this context, IL-12 producing DC were superior to similarly modified fibroblasts or to direct injection of IL-12 expressing viruses. Ag presentation by IL-12 producing DC was required for immune activation, thus suggesting *in vivo* uptake of antigen. Similarly, DC loaded with tumor Ag were more effective after transduction with IL-12- or TNFα-encoding genes [15, 103]. DC loaded with peptide Ag or genetically loaded with Ag and simultaneously transduced with co-stimulatory molecules B7.1, ICAM-1, LFA-3, or 4-1BB induced superior specific immunity compared with DC loaded with peptides only [109, 110]. Transduction with TNFα, CD40L or CD40-crosslinking antibodies likewise improved immune activation by DC, probably by improving DC maturation or mimicking $CD4^+$ T-cell help [99, 111, 112]. Similarly, transduction with GM-CSF, which was shown to improve Ag presentation, trafficking, and survival of DC, resulted in superior tumor immunity [100, 113, 114]. Finally, Bcl-X_L, a cytoplasmic, anti-apoptotic protein, enhanced survival of DC following intratumoral injection [115]. For a more complete overview of cytokine immune stimulator gene-transduced DC see [89].

In the light of these results obtained with gene-modified DC it is important to note that adenoviral gene transfer vectors feature a remarkable capacity for the insertion of heterologous DNA, which can be up to 36 kb for helper-dependent vectors. Thus, adenovirus vaccines offer the opportunity to combine (multiple) Ag loading with a transfer of various immune modifiers of different qualities.

55.2.4
Adenoviral Ag Delivery for HIV/SIV Vaccination in Monkeys

In vivo adenoviral vaccination has been assessed in depth in baboons and rhesus macaques in the context of HIV or SIV infection [116–119]. In this context, different protocols with adenoviral vaccines encoding individual or multiple HIV or SIV genes have resulted in protection against disease progression, in cleared viremia or even in protection from mucosal infection with pathogenic virus strands. Importantly, these studies demonstrated that adenoviral vaccination induced prolonged humoral and $CD8^+$ CTL-biased cellular HIV/SIV-directed immune responses. In direct comparison, adenovirus vaccination was superior to plasmid or *Vaccinia* virus vaccines. Also, multigene vaccines elicited superior immune activation than vaccination with individual genes. Anti-adenovirus antibodies attenuated but did not abolish immune responses in non-human primates. Adverse effects by anti-Ad antibodies were addressed successfully by boosting with increased virus titers. In addition, subsequent injections of different adenovirus serotypes that do not induce cross-neutralizing antibodies were reported to be effective [120]. Combination regimens with adenovirus booster injection after primary plasmid vaccination or protein/peptide booster after primary adenovirus vaccination resulted in effective immune activation. Such protocols indicate that repeated adenovirus application in patients might be unnecessary. These studies, however, did not reveal the cellular basis for Ag presentation and T-cell activation and the role of DC in this process. An understanding of the cellular and molecular mechanisms of immune induction after *in vivo* adenovirus injection, however, is pivotal for the engineering of adenovirus vectors aiming at improved *in vivo* adenovirus vaccines. DC vaccination after *ex vivo* adenoviral transduction of the SIV gag gene and subsequent intradermal or intranodal injection of transduced DC has resulted in $CD8^+$ T-cell activation in rhesus macaques [121]. This study also revealed a lack of anti-adenoviral cellular immunity or adverse effects on DC migration after repeated vaccinations and thus confirms in non-human primates previous results from studies in mice.

Important future endeavors in adenoviral vaccination research will be to engineer advanced adenovirus vaccines and in parallel to examine their performance in patients. The former will benefit from the enormous potential of adenovirus vectors to allow for molecular modifications tailored to their therapeutic purpose. In this regard, the latest understanding of molecular and cellular immune activation can be translated into adenovirus vaccine development. Examples are the exploitation of novel DC markers for vector targeting and the expression of immune modulators to intercede with immune activation in a defined manner. On the other hand, clinical translation can capitalize on the experience gained from previous DC vaccination trials and also on recent advances in technology for monitoring of cellular and molecular immune responses in patients.

55.3
Antigen Delivery to DC by Transfection of Nucleic Acids

Recently transfection of nucleic acids coding for tumor associated Ag (TAA) or viral Ag into DC has been used as a non-viral method of Ag-loading of DC (Fig. 55.1). Transfection of nucleic acids can again be subdivided into several different methods, e.g. passive pulsing (nucleic acids are taken up by the DC using its own biological machinery), electroporation (EP) (nucleic acids are moved from one side of a cuvette to the other by taking advantage of their electric charge, and thereby crossing the cell membranes of DC present in the cuvette), and lipofection (nucleic acids are actively transported into the DC, by mechanisms not yet completely understood, using lipid-based reagents). In general two types of nucleic acids can be used: DNA or RNA; both with individual advantages and disadvantages. Transfer of DNA to DC falls under the definition of gene therapy (i.e. an experimental procedure aimed at replacing, manipulating, or supplementing nonfunctional or malfunctioning genes with healthy genes), and is, in contrast to RNA transfer, subject to much stricter regulatory issues. Transfer of DNA would in theory allow for effective protein expression, since many mRNA-copies can be transcribed from one DNA-copy. In addition, DNA is more stable than RNA, allowing prolonged protein expression within the DC. Alternatively, transfer of RNA to DC falls under the definition of genetic engineering (i.e. altering the genetic material of cells or organisms to enable them to make new substances or perform new functions), and compared with DNA, mRNA has the large advantage that there is no risk of integration into the host genome.

Transfection of nucleic acids into DC was developed as an alternative strategy for Ag loading next to peptide loading, and has several different advantages (see above). Moreover, nucleic acids have several advantages compared with viruses when used for Ag delivery: (i) by selection of nucleic acids, only genes of interest are transcribed (i.e. no viral genes are co-expressed); (ii) no insertion of foreign DNA into the genome of the DC will take place when using RNA; and (iii) there is no need for cell proliferation to deliver the Ag, which is necessary for many viruses to infect cells [122].

Until recently, the largest disadvantage of transfection of nucleic acids into DC for Ag delivery was that, compared with viral gene transfer methods, only low efficiencies of gene transfer, Ag expression and subsequent presentation were obtained. Here we will describe and compare the applicability and optimization of three different methods for nucleic acid transfection (passive pulsing, EP, lipofection) into mostly human monocyte-derived DC (Mo-DC) [27, 30, 122–127], since these have been optimized for clinical use [128–130], but also other types of DC, such as human CD34 progenitor-derived Langerhans cells [27, 124], human CD34 progenitor-derived DC [27, 124], and mouse bone-marrow derived DC (BM-DC) [131].

55.3.1 Passive Pulsing

The first report on passive pulsing of mRNA was published in 1998, and described the passive transfer of mRNA coding for carcino-embryonic Ag (CEA) into immature human Mo-DC in medium alone. These RNA-transfected DC were able to stimulate a CEA-specific CTL response which was comparable to that stimulated with DC transfected in the presence of lipid [132]. Notwithstanding, the exact mechanism by which mRNA enters the DC during passive pulsing is still unknown. Most probably, mRNA is taken up by the DC using its own biological machinery. Macropinocytosis, a non-receptor-mediated process during which vesicles containing extracellular fluid with solutes are internalized by the DC, is the most likely candidate for RNA uptake via passive pulsing [133]. This process is most efficient at the immature stage of DC and not at the mature stage [134].

Compared with other methods of transfection (i.e. EP and lipofection as described below) the advantage of passive pulsing is that this method is relatively harmless to the DC (i.e. low cell mortality). However, passive pulsing is accompanied by very low transfection efficiencies. Expression of the reporter gene GFP/EGFP could hardly, or not at all be measured after passive pulsing of mRNA into differently generated DC (e.g. immature transfected Mo-DC [124], Mo-DC matured with TNFα/LPS [124], Mo-DC matured with TNFα/IFNα [125], human CD34 progenitor-derived LC, human CD34 progenitor-derived DC [124], and immature Mo-DC which were matured after transfection with poly(I:C) [126]). Furthermore, influenza membrane protein 1 (IMP-1) was hardly detected by PCR after passive pulsing of mRNA into Mo-DC [135].

Nevertheless, several papers have been published describing preclinical studies using passive pulsing of mRNA into DC which then were able to induce specific CTL response against prostate-specific Ag (PSA) and telomerase reverse transcriptase (TERT) [136, 137]. Moreover, the use of DC transfected with total tumor RNA of renal cell carcinoma tissue [138] or total amplified tumor RNA [139] of prostate cancer tissue [140], resulted in the induction of polyclonal CTL responses against several known (TERT and PSA+TERT, respectively) and unknown tumor Ag.

Gilboa and Vieweg postulated that this discrepancy between the amount of expressed and presented Ag and the stimulatory capacity of DC might be a reflection of the sensitivity of the immune system to recognize small amounts of Ag. T cells might be activated by a very low number of MHC/peptide complexes presented on the surface of DC, and increasing the amount of Ag in the DC might even be counter-productive, e.g. by inducing activation induced cell death of high avidity T cells automatically selecting for lower avidity T cells [141].

Based on the aforementioned *in vitro* studies, two clinical trials using DC passively pulsed with RNA coding for PSA [142] or total renal cell carcinoma RNA [143] were conducted. No evidence of dose-limiting toxicity or adverse effects, including autoimmunity, was seen in both trials. Furthermore, tumor-specific T cells were expanded after immunization [142, 143].

In sharp contrast to the data described above, several other groups found it hard to induce any T-cell response using DC passively pulsed with mRNA. No IFNγ release by MelanA-specific CTL clones was seen after stimulation with several DC populations (e.g. TNFα/LPS matured Mo-DC, immature Mo-DC, human CD34 progenitor-derived LC, human CD34 progenitor-derived DC [124], and immature transfected and then poly(I:C)-matured Mo-DC [126]) passively pulsed with MelanA RNA. Furthermore, Van Meirvenne and co-workers showed that murine BM-DC passively pulsed with Ii80.tOVA (i.e. an RNA coding for ovalbumin containing a part of the murine invariant chain (Ii) that targets the encoded protein towards the endolysosomal pathway, enabling class I and class II presentation) were not able to induce IL-2 production by MHC class I/OVA and MHC class II/OVA restricted hybridomas [131].

Most importantly, induction of a memory response against IMP [135], and induction of primary CTL responses against MelanA [126, 135] were unsuccessful when using passively pulsed DC. In all the cases described above, DC electroporated with the same mRNA were able to induce T-cell responses, indicating that the amount of Ag in the DC did have a positive effect on the immunostimulatory capacity of the DC. Moreover, in preliminary experiments we were able to generate DC populations with a different Ag density per DC and we clearly saw that those DC with a high Ag density were much better at stimulating MelanA-specific $CD8^+$ T cells compared with DC with a lower Ag-density (Dörrie and Schaft, manuscript in preparation). Since most tumor Ag are also expressed, although to a lower extent, by normal tissue, it might even be beneficial initially to use DC expressing and presenting much Ag on their surface to break the tolerance against these tumor Ag and induce a vigorous T-cell response. Subsequent vaccination with DC with a lower Ag density per cell could then be used to maintain this T-cell response and to avoid activation induced cell death.

55.3.2
Electroporation

Recently, EP of nucleic acids into DC was studied and optimized by several groups [27, 123–125]. One of the largest advantages of EP of nucleic acids compared with lipofection (see below) is that it does not require additional reagents and that it is compatible with clinical use, which is often not the case for lipofection reagents.

Initial experiments concentrated on EP of plasmid DNA (pDNA), coding for the reporter gene EGFP, into several different DC populations, such as human CD34 progenitor-derived LC, human CD34 progenitor-derived DC, and Mo-DC [27]. Although the transfection efficiency into human CD34 progenitor-derived LC and human CD34 progenitor-derived DC was reasonable (i.e. 16% and 12%, respectively), Mo-DC were quite refractory to EP with pDNA (i.e. 2% transfection efficiency) [27]. These low levels of gene transfection by EP of pDNA were in line with data of Arthur et al. [23]. Furthermore, pDNA transfection into DC was accompanied by loss of DC phenotype and a rapid decrease in cell viability [23, 27]. However, EP of pDNA did not affect the allo-stimulatory capacity of DC [27, 123]. The transfection

efficiency of (mature) Mo-DC with pDNA was increased to 15% by Saeboe-Larssen and co-workers using square-wave EP, but yielded also a low survival rate (i.e. 50%) [125]. These low survival rates after EP of pDNA into DC can be explained by the electrical settings used for EP. pDNA has to reach the nucleus for efficient expression of the incorporated gene, and thus pDNA has to cross both the cytoplasmic and the nuclear membrane. Therefore, relatively high voltages and/or EP times have to be used, resulting in cytotoxicity [27, 125].

After these first experiences with EP of pDNA, several research groups started to compare EP of mRNA with EP of pDNA coding for the same gene. Van Tendeloo et al. first carefully compared EP of EGFP encoding pDNA and EGFP encoding mRNA into leukemic K562 cells [124]. pDNA EP was less efficient than mRNA EP as measured by the number of transfected cells [124]. However, questions were raised about the stability of gene expression in mRNA electroporated cells, since mRNA seemed to be very labile. Importantly, FACS analysis revealed that the level and duration of EGFP expression after mRNA EP was in the same range as the level and duration of EGFP expression after pDNA EP [144].

Furthermore, EP experiments with DC showed that EP of mRNA was more effective than pDNA [123–125]. Since mRNA has only to cross the cytoplasmic membrane to be translated by ribosomes, EP of mRNA resulted in high transfection efficiency, less cytotoxicity (probably because of milder electrical settings), and rapid expression of protein. An additional advantage of mRNA is that it is easy to produce from *in vitro* transcription vectors using appropriate primers and reverse transcriptase-polymerase chain reaction (RT-PCR) [139, 145]. Moreover, EP of mRNA compared with pDNA is highly reproducible, easy to perform, and does not affect the DC phenotype or viability [146].

In contrast to DC passively pulsed with mRNA, mRNA-electroporated DC represented an effective tool to stimulate T-cell clones specific for MAGE-3 [30, 45, 147], MAGE-1 [144], and MelanA [30, 124, 126]. Interestingly, Van Tendeloo observed a non-specific stimulation of the MelanA-specific CTL clone by DNA electroporated DC, which was not observed with RNA-electroporated DC [124]. Furthermore, MHC class I/OVA and MHC class II/OVA restricted hybridomas were very efficiently stimulated by Ii80.tOVA mRNA-electroporated DC [45, 131].

Moreover, DC electroporated with mRNA encoding a broad range of antigenic targets have been used to stimulate T-cell responses *in vitro* against viral proteins, such as IMP (memory response) [127, 135, 148], as well as tumor Ag, such as MelanA [30, 126, 135], human TERT [125], myeloma Ag [149], and renal cell carcinoma Ag [150]. Even *in vivo* T-cell responses against OVA were seen after immunization with mouse BM-DC which were electroporated with Ii80tOVA RNA [45], and a clinical trial is underway in which patients with prostate cancer are vaccinated with human TERT mRNA-electroporated DC [141].

Although RNA-electroporated DC are clearly able to induce immune responses, there is still an urgent need to use well-characterized, standardized, and validated DC vaccines [151]. Several parameters, such as optimal Ag loading, need to be addressed systematically first by careful preclinical research and then in (preferably two-armed) clinical trials [14, 151]. In most of the studies described above, mRNA

was electroporated into immature Mo-DC, which were then matured. However, the use of mature DC would be more straightforward from a handling point of view (i.e. less manipulation of the cells, less problems with stickiness). Nonetheless, an initial report showed a lower transfection efficiency of mature DC [124], and concerns were raised that DC transfected following maturation will only present epitopes generated by the immunoproteasome but not standard proteasome-dependent ones. In contrast to passive pulsing, which is most efficient at the immature stage of DC, it was not clear whether it would be better to mature DC before or after RNA EP.

We carefully compared EP of RNA coding for three different Ag (i.e. MelanA, MAGE-3, and Survivin) before or after maturation of DC. We found that immature and mature DC, using appropriate methods, can be electroporated with equally high yield and transfection efficiency. The migration capacity of electroporated DC was in the same range as of non-electroporated DC, and Ag expression could be validated and studied with respect to kinetics by intracellular antibody staining [30]. Thus, this method creates a highly reproducible and validatible vaccine (i.e. intracellular Ag expression can be measured at the time-point of harvesting), which is most certainly not the case with DC passively pulsed with RNA. Moreover, RNA-electroporated DC could be cryopreserved without loss of transgene expression, phenotypical properties, and stimulatory capacity, which is essential when creating several batches for vaccination [30, 45, 127, 135, 147].

Furthermore, it is known that vaccination strategies using only one tumor Ag could impose selection pressure on the tumor, which could result in Ag-loss variants. The tumor cells that lost expression of that certain tumor Ag may then escape immune rejection [152–154]. Therefore, vaccination with DC expressing several tumor Ag may be advantageous for immunotherapy, particularly for priming [155]. Concerning this, vaccination with peptide-loaded DC is restricted, since the number of defined epitopes available for various Ag is still limited, and it would also be very costly to generate a polyvalent vaccine using multiple peptides. We demonstrated that it is possible to simultaneously electroporate RNA coding for three different tumor Ag without influencing each others expression and presentation [30], which might be essential to overcome the generation of Ag-loss variants (generation of CTL against three or more tumor Ag correlated with clinical response) [156].

Most importantly, DC electroporated following maturation were immunologically superior to DC electroporated at the immature stage followed by maturation, because they generated epitopes such as Mage-3.A1 and MelanA.A2.1 more efficiently [30], which was in line with data published by Bonehill et al [147]. Surprisingly, they were also clearly superior in inducing IFNγ-producing and lytic CTL, specific for MelanA, a classical standard proteasome-dependent CTL epitope.

Induction of potent $CD8^+$ CTL responses has been the main goal in DC vaccination studies, but recently emerging evidence points out that also $CD4^+$ helper cells are required for the induction of an optimal, long-lasting antitumor immune response [157, 158]. Ag coded by electroporated RNA will preferentially enter the MHC class I presentation pathway in DC and thus stimulate only $CD8^+$ T cells, and

not CD4$^+$ T cells. Targeting Ag into the MHC class II presentation pathway would greatly enhance the efficacy of an anticancer vaccine.

Several RNA-modification tricks are available to guide the translated protein into both the MHC class I and class II presentation pathways. RNA coding for the Ag is simply linked to a stretch of RNA coding for signals associated with resident endosomal or lysosomal membrane proteins (reviewed in [159]) such as: (1) a part of the invariant chain (Ii) [131, 147]; (2) a sorting signal of lysosomal associated membrane protein-1 (LAMP-1) [132, 143, 147]; and (3) a sorting signal of DC-LAMP [147]. Furthermore, transiently inhibiting the Ii expression by using antisense oligonucleotides, co-delivered with the Ag-coding mRNA, led to enhanced class II presentation and antitumor immunity in a mouse model [160].

Taken together, further preclinical work and future clinical trials have to prove whether RNA-electroporated DC are a good tool for antiviral and antitumor vaccination. Results from this work will indicate the best way to optimal and standardized vaccines inducing both CD8$^+$ and CD4$^+$ T-cell immune responses.

55.3.3 Lipofection

EP is not the only way by which DC can be transfected with nucleic acids. Both DNA and RNA can be transfected into DC using lipid-based reagents. In a process termed lipofection, these agents form complexes with the polynucleotides, which can then enter the DC via so far unknown mechanisms, and/or encapsulate the nucleic acids in liposomes, which can fuse with the cell membrane, delivering their content into the cytoplasm.

The use of DNA in lipofection strategies led to the expression of functional transgenic protein in human DC, which is rapidly degraded and presented, inducing tyrosinase-specific T-cell activation *in vitro* [161]. However, DNA lipofection resulted in a highly variable transfection efficiency, ranging from 2% to 53%, in different donors. A phase I/II clinical trial demonstrated the feasibility and safety of an autologous DNA-transfected DC vaccine, but did not induce any clinical or immunological responses [162]. For the reasons mentioned above, RNA is preferred by most researchers for DC lipofection.

Although EP is the "en vogue" method of RNA transfection into DC at the moment, the initial experiments that showed that RNA-transfected DC can elicit an immune response were done by lipofection. DC lipofected with *in vitro* transcribed RNA (IVT-RNA) coding for OVA, or with total RNA isolated from OVA-expressing tumor cell lines, were able to stimulate primary OVA-specific CTL *in vitro*. When mice were vaccinated with these DC, the animals were protected against a challenge with OVA-expressing tumors, or the size of pre-established tumors was reduced [145]. These findings also held true for *in vitro* human systems; Mo-DC lipofected with IVT-RNA coding for CEA, or with total RNA from a CEA-expressing cell line were able to induce primary cytotoxic CD8$^+$ T lymphocytes, which in turn could lyse CEA-transfected DC or CEA-expressing, tumor-derived cells. Targeting

of the Ag towards the lysosomal compartment by expressing a CEA-LAMP-1 chimeric protein resulted in the stimulation of specific $CD4^+$ T lymphocytes [132]. This could possibly provide T-cell help which is necessary for a potent immune response *in vivo*. In these early studies immature DC were used as stimulator cells, but soon it was shown that maturation of DC after transfection with Mart-1/MelanA-coding RNA highly increased the stimulatory capacity of these DC, probably due to an increased level of presentation and better co-stimulation [124, 126]. In the same two studies superiority of EP over lipofection was first shown in a direct comparison. While immature DC could be transfected with efficiencies of ≥50%, lipofection reached a mere 10% [124] or 20% [126] of the DC.

Recently protocols were developed to transfect mature DC with cationic lipids in combination with RNA-condensing agents. The transfection after maturation could be beneficial for the reasons discussed above. With these methods it was possible to transfect TNFα matured DC and LPS matured DC with a 16% and 32% efficiency, respectively [163]. DC matured with a cocktail of IL-1β, IL-6, TNFα, and PGE-2 were transfected with over 50% efficiency [164]. In the last case, efficient priming and expansion of high-affinity MelanA-specific T cells was observed. In addition, Ag presentation was stable over 4 days. Now that the technical problems of lipofection, e.g. inefficient transfection and high toxicity of the lipid-based reagents to DC, have been solved or confined, lipofection should again be considered an alternative to EP as method of efficient RNA transfection.

55.4 Concluding Remarks

Both RNA and DNA transfer into DC offer a practical and flexible tool for antigen delivery and immune modulation, resulting in advanced DC vaccines. Whereas RNA transfer is an efficient and, with respect to technology and regulatory issues, a rather uncomplicated approach for effective *ex vivo* DC-vaccination protocols, adenoviral DNA transfer embodies a means of adaptation to various vaccination strategies, including *in vivo* antigen loading and manipulation of DC, by engineering of the gene transfer vector. Nevertheless, key aspects of such vaccination strategies remain undefined and further *in vitro* and *in vivo* testing is needed for the design of an optimal vaccine.

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Color Plates

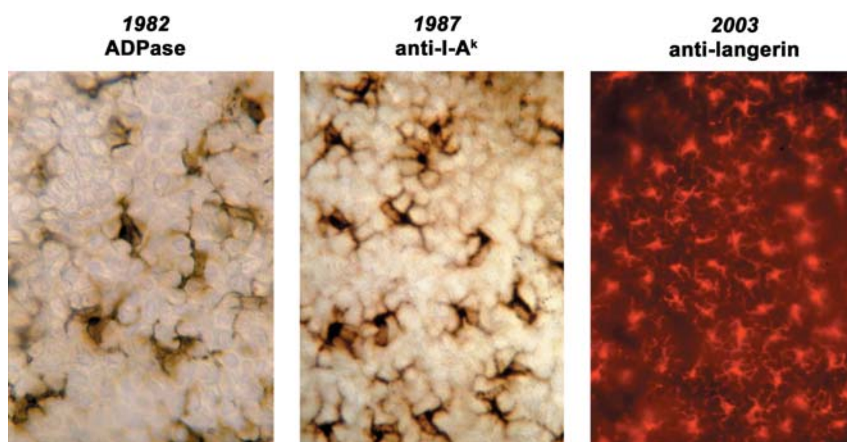

Fig. 5.1 Langerhans cells in epidermal sheet preparations, as visualized over the years. One of the first molecules that served as a signature for Langerhans cells *in situ* was ATPase or ADPase. A photo from 1982 shows the immunocytochemical visualization of Langerhans cells by means of ADPase activity. As monoclonal antibodies became available, anti-Ia (i.e. anti-MHC class II) antibodies were widely used to detect Langerhans cells in the epidermis. This is depicted in the photo from 1987 using an immunoperoxidase detection technique. More recently, the langerin/CD207 molecule was recognized as the most selective Langerhans cell marker. The picture from 2003 shows mouse Langerhans cells *in situ*, detected by monoclonal anti-langerin antibody using an immunofluorescence technique. Note that the magnifications are not equal.

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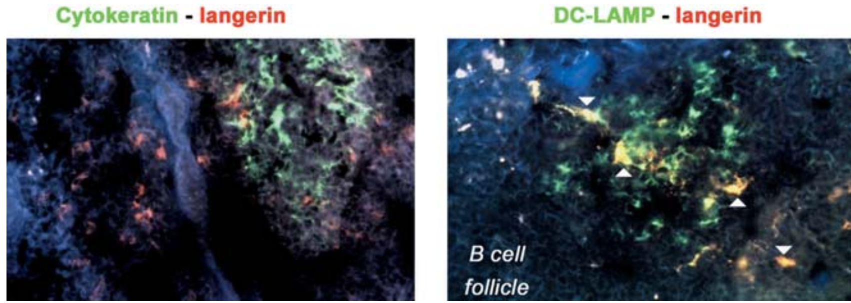


Fig. 5.3 Langerhans cells in the human tonsil. Cryosections of human tonsils were immunolabeled with monoclonal antibody DC-GM4 against human Langerin (red fluorescence). Left: the green fluorescence of double-labeling with antibody against cytokeratin shows that few Langerhans cells (red cells) are located within the epithelium of the tonsillar crypt, most Langerhans cells being found in the T-cell area. Right: in the T-cell area virtually all Langerhans cells express the maturation marker DC-LAMP (i.e. yellow/ orange fluorescence). Many mature non-Langerhans cells are present (green fluorescence of DC-LAMP$^+$/langerin$^-$ cells). Immature Langerhans cells (red fluorescence of DC-LAMP$^-$/langerin$^+$ cells) cannot be spotted in the T-cell zone of the tonsil.

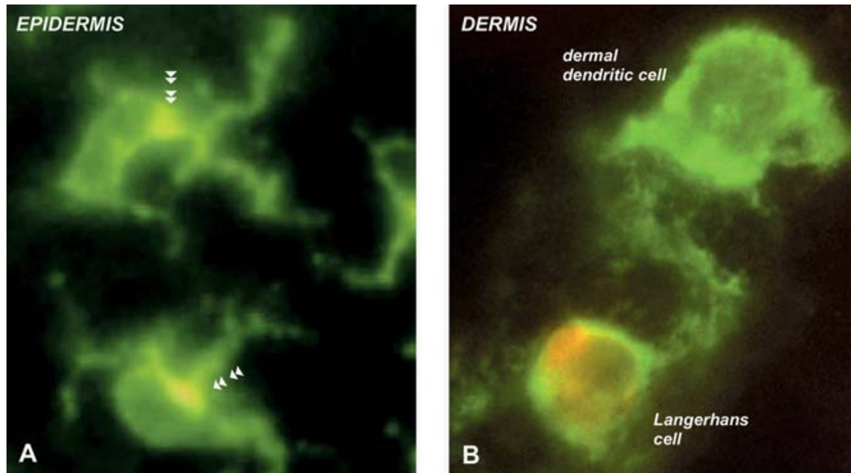


Fig. 5.4 Translocation of MHCII molecules to the cell surface during Langerhans cell maturation in the mouse. Immunofluorescence using anti-MHC II monoclonal antibody (green fluorescence) was performed on epidermal sheets of untreated skin (A) and on a dermal sheet of a whole skin explant that had been cultured for 48 h (B). The two cells in B are located in a lymph vessel ("cord"). The migrating Langerhans cell was additionally identified by its expression of langerin (red fluorescence). Even by conventional fluorescence microscopy it becomes evident that Langerhans cells *in situ* have most MHCII molecules concentrated in a bright intracellular spot (arrows in A). During migration and concomitant maturation most MHCII becomes translocated to the cell surface and thus appears as a brightly fluorescing rim. This applies both to the Langerhans cell and to the langerin-negative dermal dendritic cell travelling through the lymph vessel (B).

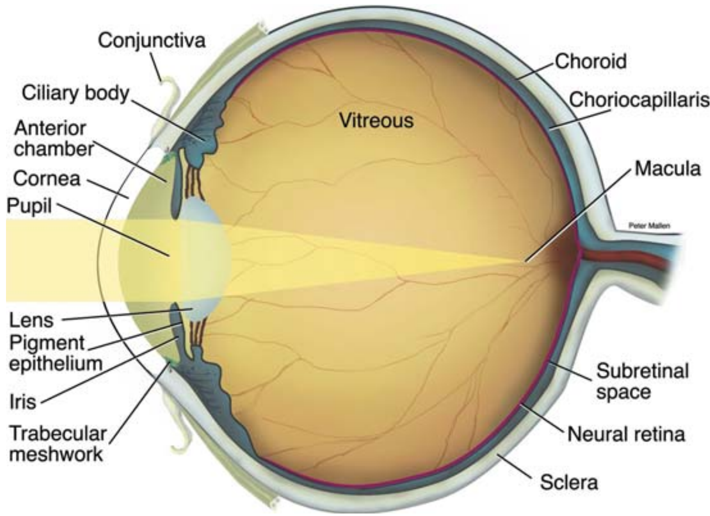


Fig. 6.1 Anatomy of the eye. The illustration identifies the anatomical parts of the eye and shows how light focuses on the macula or center of the retina.

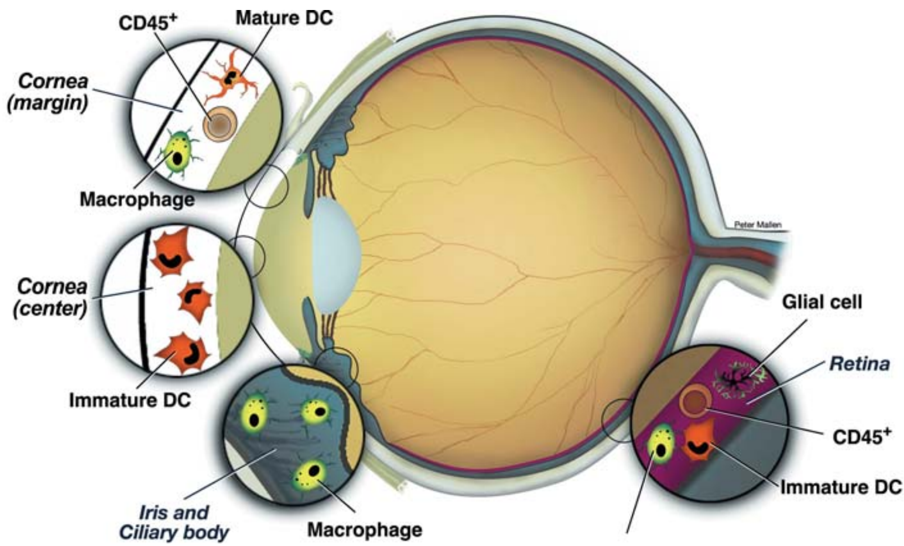


Fig. 6.2 APC in the eye. The illustration identifies the cells in the eye by marker and location. Retina: CD45+ and CD45+ CD11b, F4/80+ cells have been studied in the retina. Dendritic like cells have not been observed in this region. Cornea: A variety of potential APC are in the cornea and differentiated by their expression of surface markers and their location in the various regions of the cornea.

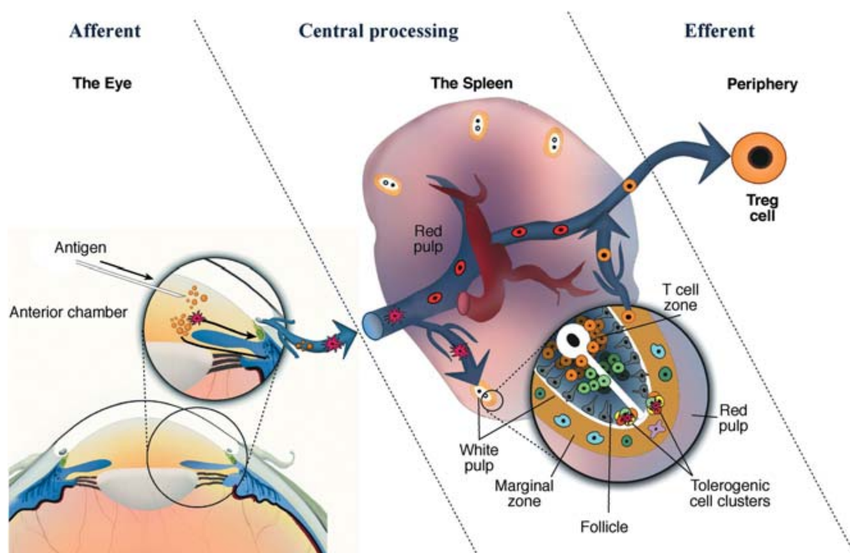


Fig. 6.3 Immune reflex arc of tolerance. The illustration shows three stages of mechanisms involved in the induction of peripheral tolerance in ACAID. Antigen is presented to the anterior chamber, where it is picked up by the F4/80+ APC located in the trabecular meshwork, ciliary body or the cornea. The antigen-loaded APC then leave the tissue via the trabecular meshwork and travel to the blood to the marginal zone of the spleen where they interact with other cells to produce Treg cells. Treg cells then travel to the periphery where they function as afferent and efferent T suppressor cells.

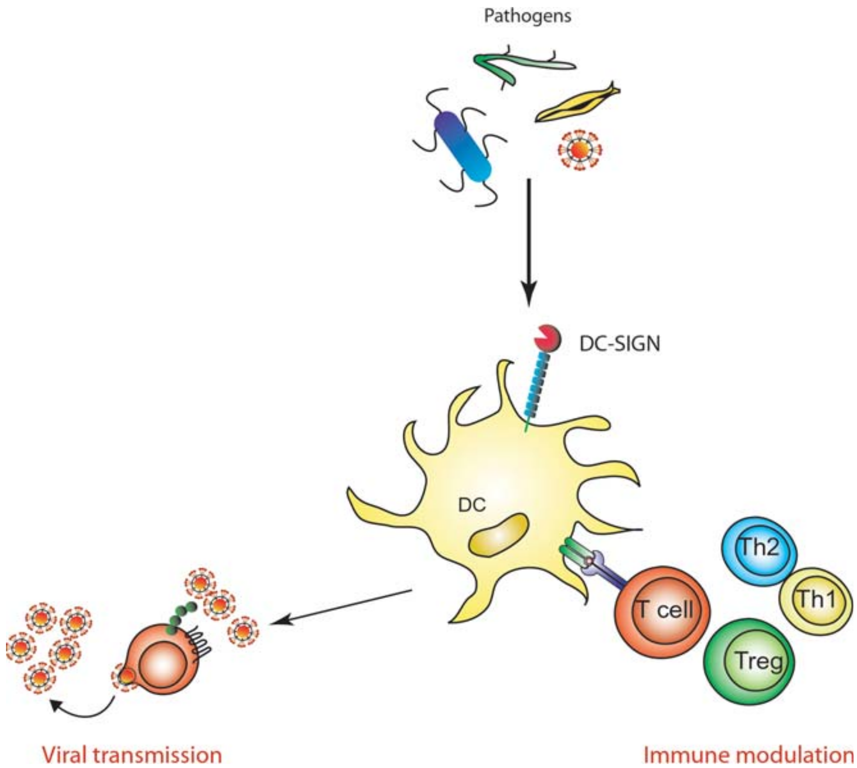


Fig. 8.1 The initial interaction of pathogens with DCs determines the immunological outcome. CLRs such as the C-type lectin DC-SIGN recognizes different pathogens through their carbohydrate structures. Although many pathogens interact with this receptor, it is becoming evident that the immunological outcome of this interaction is specific for the pathogen. DC-SIGN might cooperate with other innate immune receptors, such as Toll-like receptors to fine-tune the immune responses.

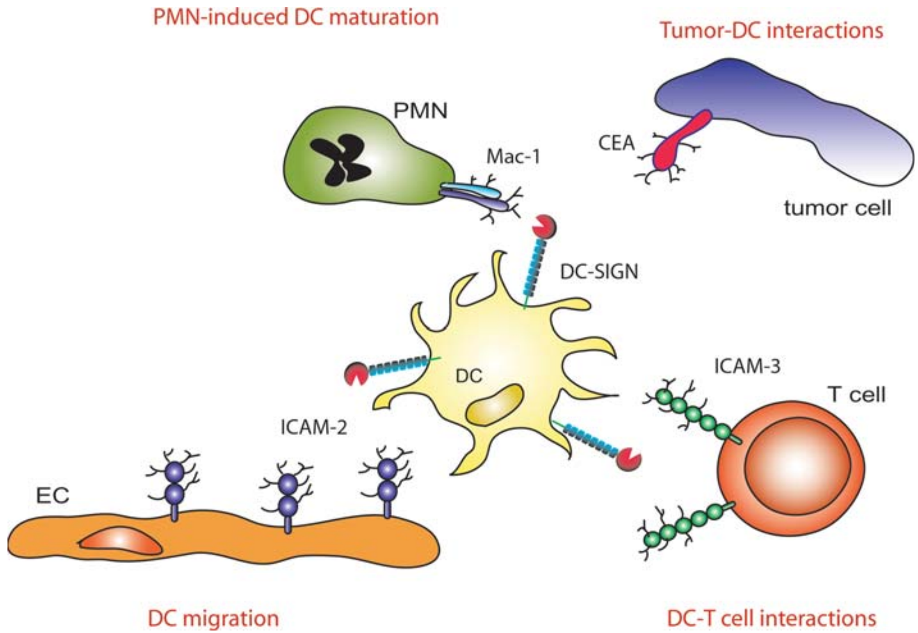


Fig. 8.2 C-type lectins govern cellular communications that may modulate adaptive immune responses. CLRs such as the C-type lectin DC-SIGN functions as an adhesion receptor by facilitating Cell-cell interactions upon recognition of glycan structures on self glycoproteins. DC-SIGN facilitates trans-endothelial migration of DCs through ICAM-2 and initiates DC-T cell interactions through recognition of ICAM-3 which allow scanning of the presented MHC-peptides complexes by T cells. Activated neutrophils strongly cluster with DCs, which modulates DC-induced immune responses, thus providing a link between innate and adaptive immune responses. Upon malignant transformation, colon epithelial cells change their glycosylation, resulting in an altered glycosylation of tumor-specific antigens such as CEA. Tumor-derived CEA, but not normal CEA, is recognized by DC-SIGN on DCs, which might result in protective immune responses or allows unbridled growth of the tumor.

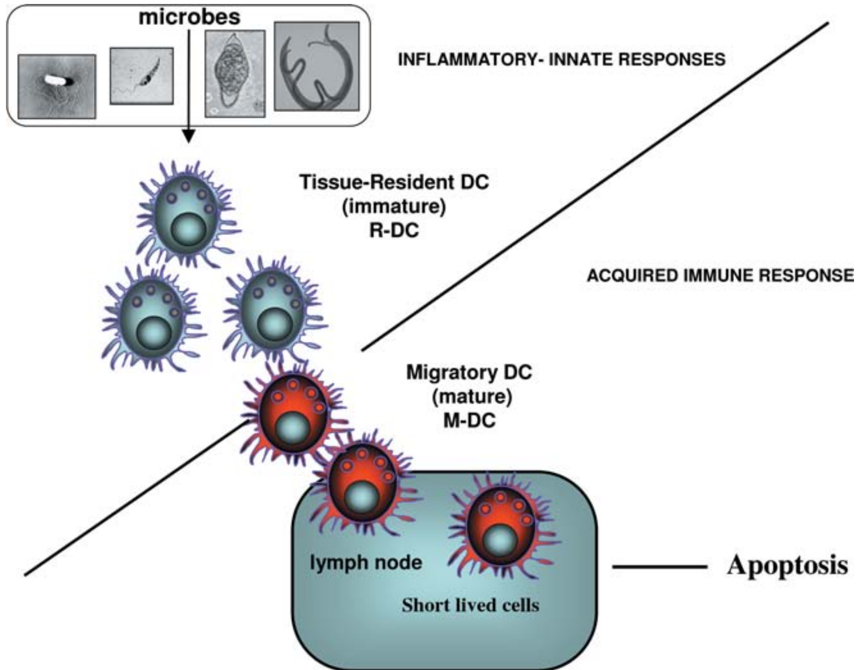


Fig. 11.1 Dendritic cells (DCs) can be divided into two main subpopulations: the resident dendritic cells (R-DC) found in the tissues where they are seeded and the migratory dendritic cells (M-DC) that will migrate to the lymph node upon antigen encounter. Immature R-DC are strategically located in tissues that represent pathogen entry routes, where they continuously monitor the environment through the uptake of both particulate and soluble products. DC maturation is associated with acquisition of migratory functions that allow antigen-loaded DC to move from the marginal zones to the T-cell areas or from nonlymphoid to lymphoid tissues. Mature migratory DC have abundant cell surface major histocompatibility complex (MHC) and co-stimulatory protein expression, have the ability to activate both CD8 and CD4 T cells and undergo cytoskeleton rearrangements that lead to the inhibition of the phagocytic activity and are programmed for apoptotic death.

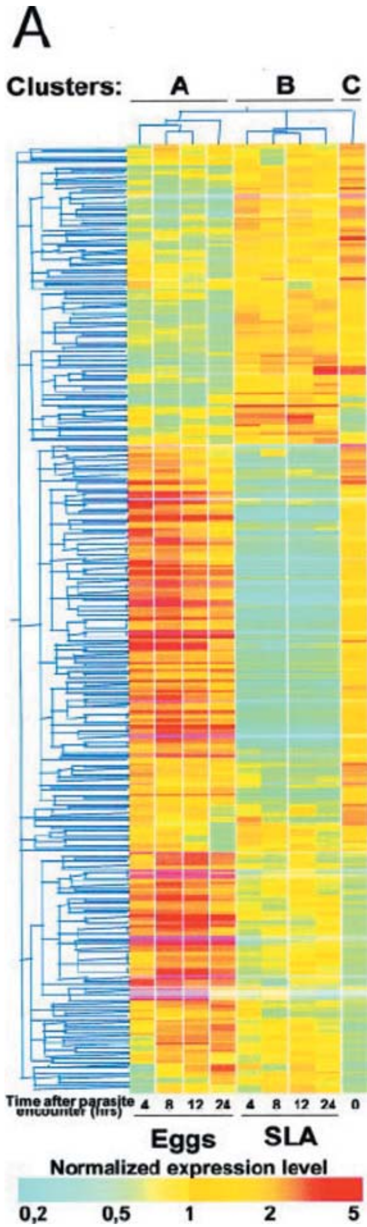


Fig. 11.3 Expression profile clustering of 283 genes differentially expressed during DC–schistosome interaction. Two-way hierarchical clustering of gene expression profiles measured in time-course experiments; normalized expression levels relative to median are displayed in yellow (median expression), red (increased expression), or cyan (decreased expression) according to the color bar.

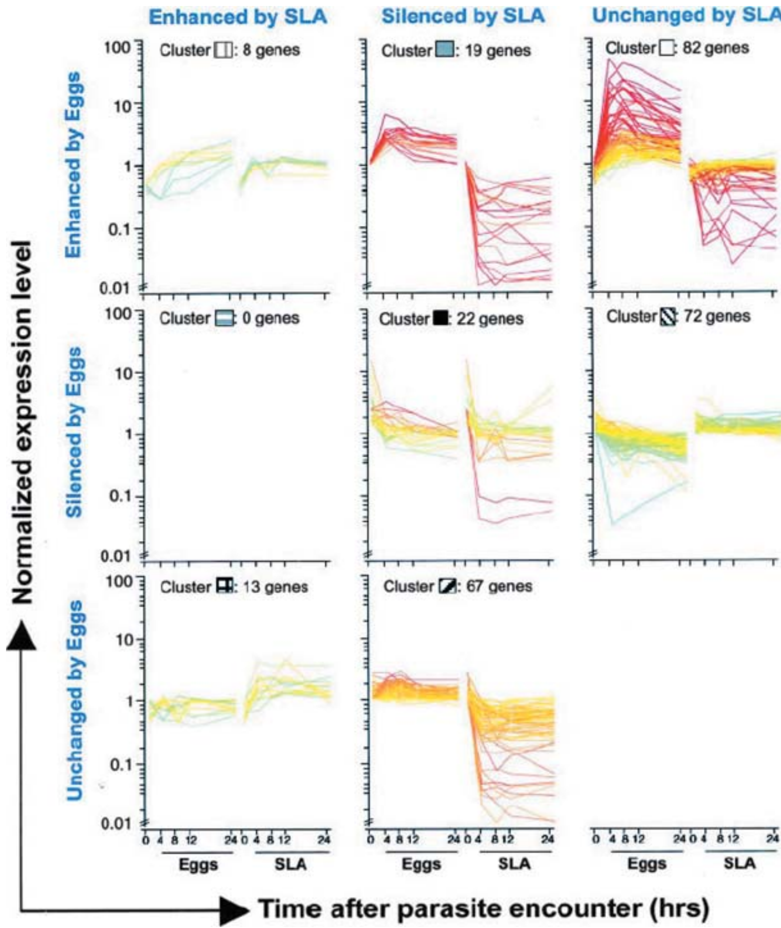


Fig. 11.4 Expression profile clustering of 283 genes differentially expressed during DC–schistosome interaction. Supervised clustering of kinetic gene expression profiles. Each panel groups genes that share same transcriptional response (enhanced, silenced or unchanged) relative to the two developmental stages of the parasite. Each line represents the expression profile of a particular gene and is colored according to its normalized expression level 4 h after encountering *S. mansoni* eggs.

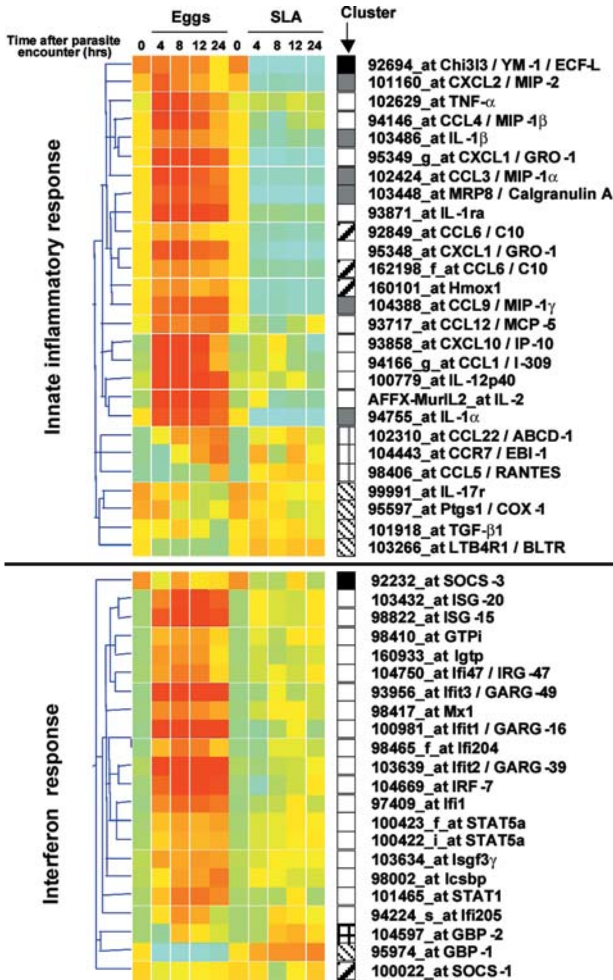
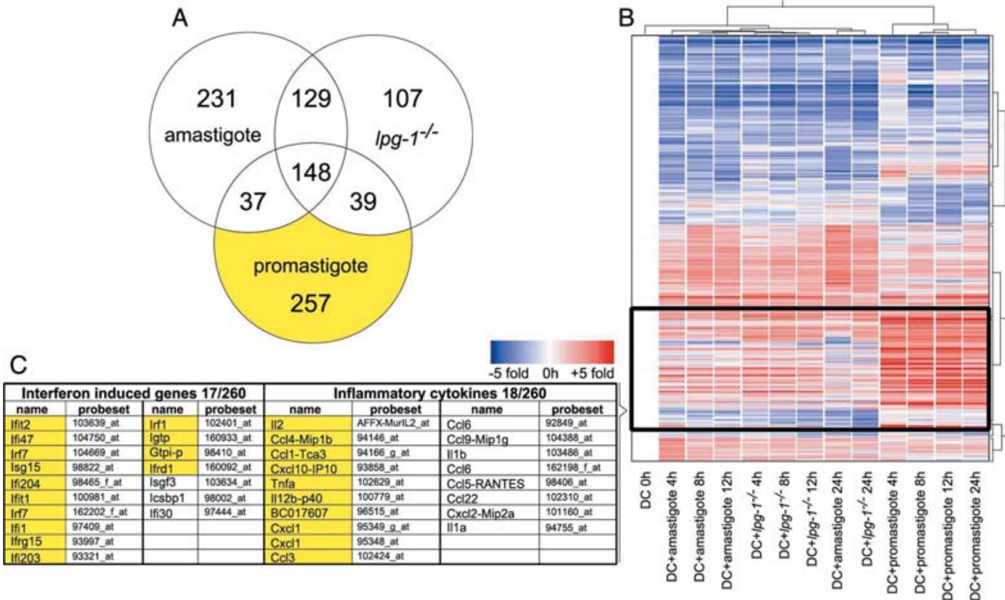


Fig. 11.5 Functional classification of differentially expressed genes. Each colored box represents the normalized expression level of a given gene in a particular experimental condition and is colored according to the color bar. Interferon-induced genes are shown.



Interferon induced genes 17/260				Inflammatory cytokines 18/260			
name	probeset	name	probeset	name	probeset	name	probeset
Ifit2	103639_at	Irf1	102401_at	Il2	AFFX-MurIL2_at	Ccl6	92849_at
Ifi47	104750_at	Igtp	160933_at	Ccl4-Mip1b	94146_at	Ccl9-Mip1g	104388_at
Irf7	104669_at	Gtpi-p	98410_at	Ccl1-Tca3	94166_g_at	Il1b	103486_at
Isg15	98822_at	Ifrd1	160092_at	Cxcl10-IP10	93858_at	Ccl6	162198_f_at
Ifi204	98465_f_at	Isgf3	103634_at	Tnfa	102629_at	Ccl5-RANTES	98406_at
Ifit1	100981_at	Icsbp1	98002_at	Il12b-p40	100779_at	Ccl22	102310_at
Irf7	162202_f_at	Ifi30	97444_at	BC017607	96515_at	Cxcl2-Mip2a	101160_at
Ifi1	97409_at			Cxcl1	95349_g_at	Il1a	94755_at
Ifrg15	93997_at			Cxcl1	95348_at		
Ifi203	93321_at			Ccl3	102424_at		

Fig. 11.6 *L. mexicana* promastigotes expressing LPG trigger a pro-inflammatory transcriptional response in D1 cells. (A) A total of 948 differentially expressed mRNAwere organized according to common or specific modulation in D1 cells infected with *L. mexicana* amastigotes or wild-type or lpg1–/– mutant promastigotes (lpg1–/–). (B) Hierarchical clustering according to samples (vertical) and gene expression profiles (horizontal). Mean gene expression values are shown, and gene signals are divided by their 0-h value. Red and blue colors indicate up- or downregulation, respectively, in comparison to 0-h time point values (white). A cluster of 260 genes that contains all differentially expressed genes belonging to interferon-induced genes ($n = 17$) and inflammatory cytokine genes ($n = 18$) is boxed. (C) These genes, which belong to the promastigote-specific group of the Venn diagram (A), are color-coded in yellow.

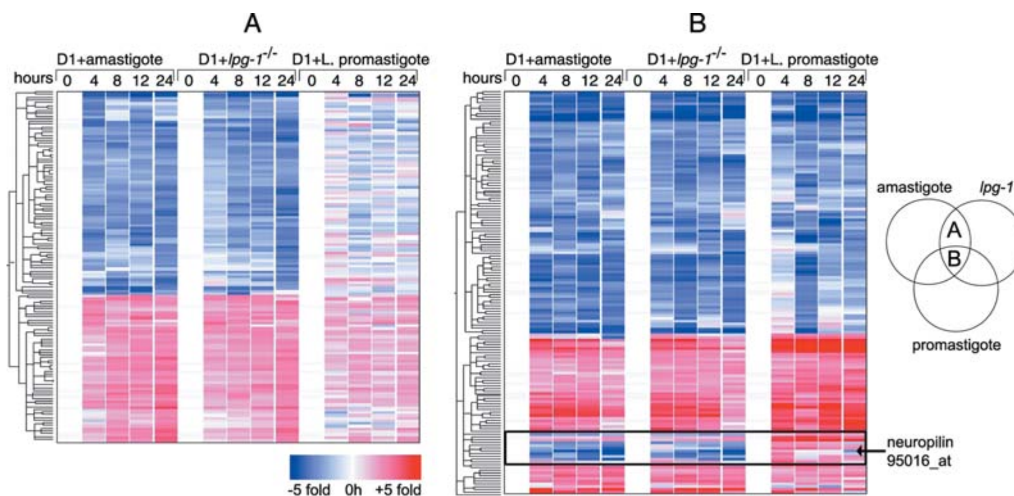


Fig. 11.7 Hierarchical clustering of commonly modulated mRNA reveals an LPG-induced signature in D1 cells. The expression profiles of 129 genes modulated in D1 cells after infection with either amastigotes or lpg1–/– mutants is represented in (A), whereas the expression profiles of 148 genes modulated by all three parasite forms is represented in (B) Time course profiles are shown horizontally. The black box highlights a new part of the LPG signature, and neuropilin is indicated (see text for details). Red and blue colors indicate up- and downregulation, respectively, in comparison to 0-h time point values (white).

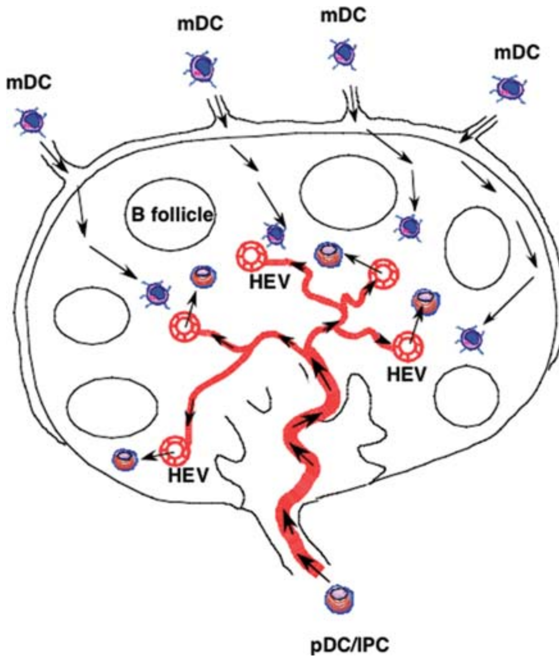


Fig 13.1 Migration pathways of pDC/IPCs versus mDC into a lymph node. pDC/IPC migrate into a lymph node through blood and high endothelial venules (HEV). mDC migrate into a lymph node through afferent lymphatics. Both pDC/IPC and mDC are localized in the T cell rich-areas.

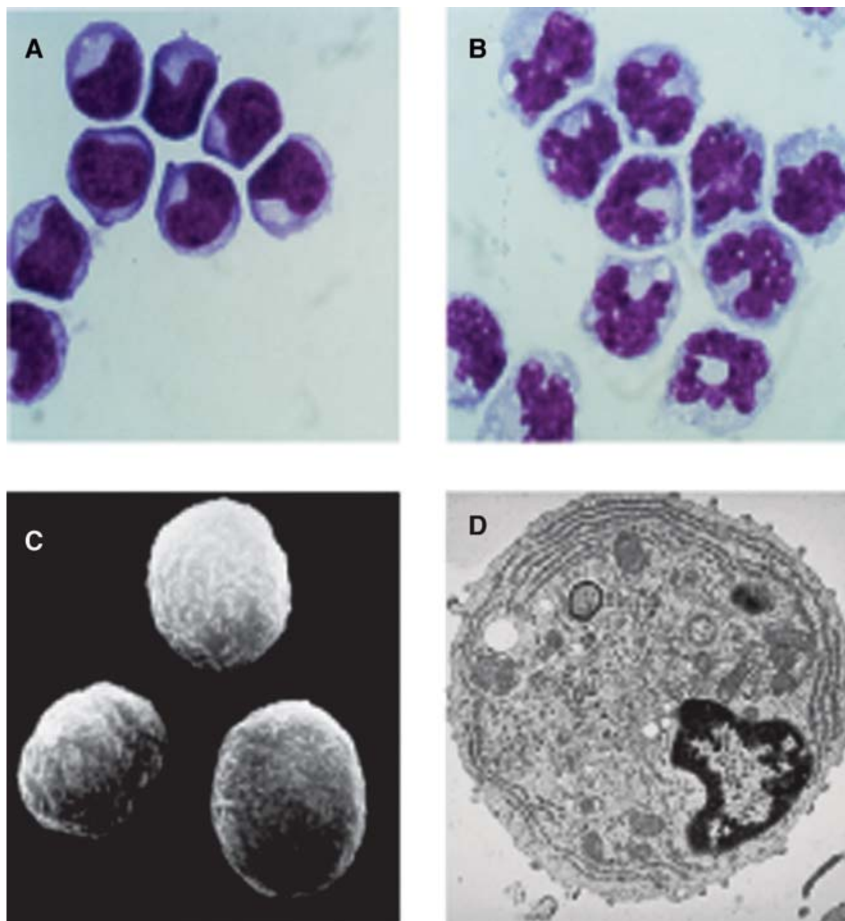


Fig 13.2 Morphology of pDC/IPC. A. Giesma staining of pDC/IPC; B. Giemsa staining of monocytes; C. Scanning EM of pDC/IPC; D. Transmission EM of pDC/IPC.

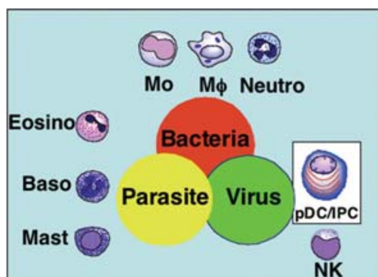


Fig 13.4 The innate immune system has dedicated cell types to control infections by three major microbes.

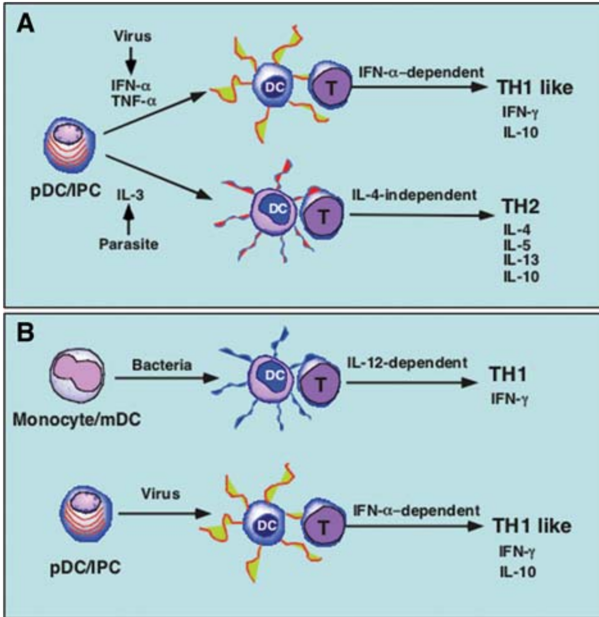


Fig 13.5 A. Functional plasticity of pDC/IPC-derived DCs. Upon viral infection, pDC differentiate into DCs mediated by autocrine IFN-α and TNF-α, which prime naïve $CD4^+$ T cells to produce IFN-γ and IL-10. Upon parasite infection, pDC differentiate into DCs mediated by paracrine IL-3 released by mast cells, eosinophils and basophils, which prime naïve CD4+ T cells to produce TH2 cytokines IL-4, 5, 10 and 13. B**.** There are two distinct antigen-presenting cell systems in humans that have the capacity to prime naïve $CD4^+$ T cells to produce IFN-γ, while bacteria-activated mDC mainly use IL-12, viral induced pDCs-derived DC use type 1 IFN.

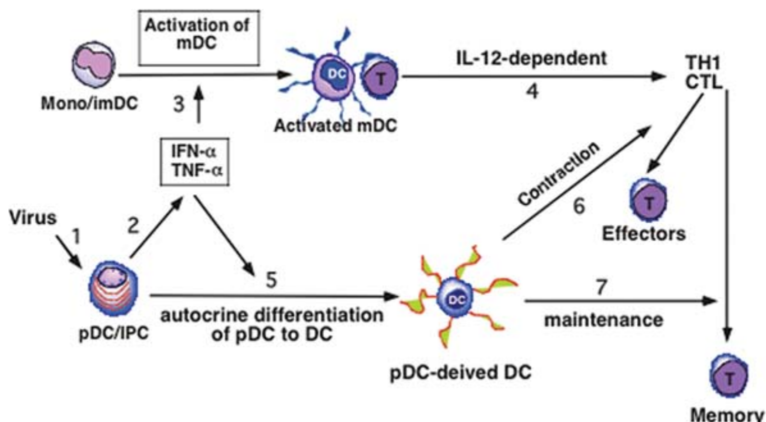


Fig 13.6 pDC/IPC regulate the function of mDC. Viral infection (1) induces pDC/IPC to produce type 1 IFN and TNF-α (2), which activate monocytes or mDC (3) to upregulate co-stimulatory molecules and secrete IL-12; the activated mDC induce strong TH1 and CTL responses (4); pDC/IPC differentiate into mature DC by autocrine IFN-α and TNF-α (5); pDC-derived DCs prime naïve T cells to produce IL-10, which may contribute to the contraction of the effector phase of T-cell responses (6); pDC-derived DCs may also enhance the generation and maintenance of memory T cells through type 1 IFN and other mechanisms (7).

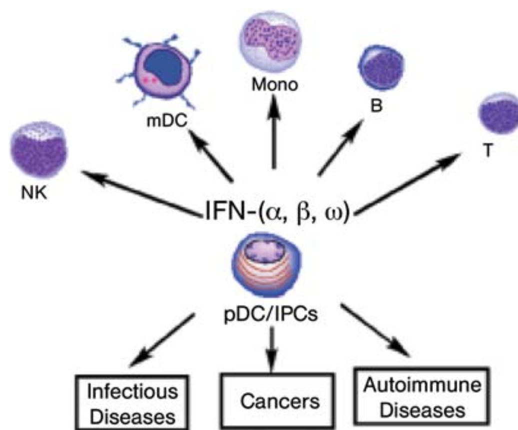


Fig 13.7 The function of pDC/IPCs in regulating the functions of other immune cells and in the control and pathogenesis of human diseases.

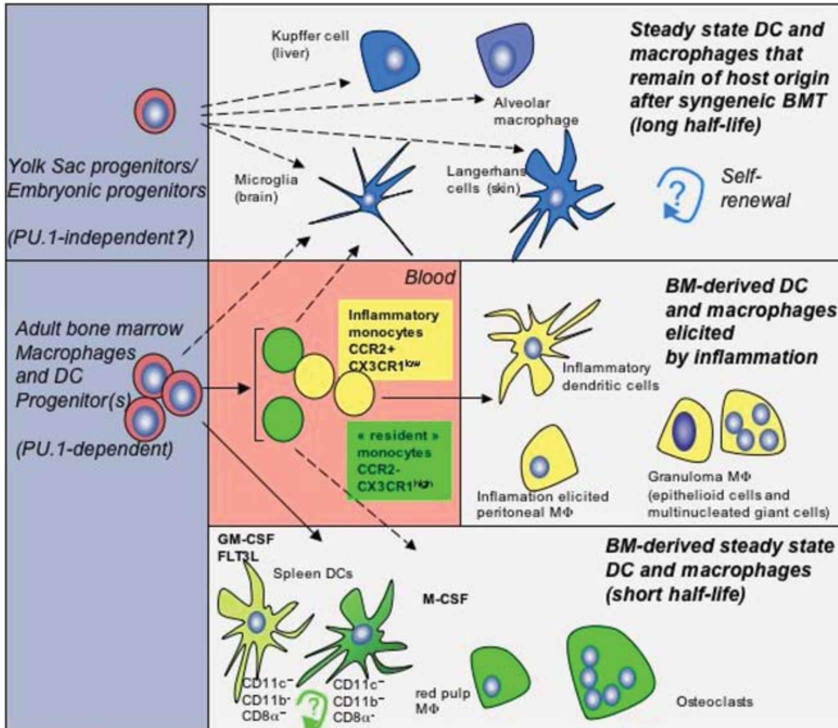


Fig. 14.1 Tentative topology of the mononuclear phagocyte system: Contribution of monocytes to the renewal of tissue macrophages and dendritic cells. Blood monocytes are divided into two main subsets. Tissues macrophages and DC can be divided in three compartments according to their half-life, their reconstitution from donor-derived bone marrow cells after syngeneic bone marrow transplantation, and the role of inflammation in their differentiation. The lineage relationship between precursors and differentiated cell types, and the established and potential contribution of blood monocyte to each of the cell types are indicated by solid and dashed arrows. Steady state macrophages and DC that remain of host origin after syngeneic bone marrow graft (colored in blue) such as LC or microglia, have a long half-life and may self renew in the steady state. Some subsets may originate from yolk sack progenitors. The contribution of bone marrow derived cells, such as monocytes, to their renewal in pathological condition remains to be established. Evidence is presented in the text that $CCR2^{+}$ $CX3CR1^{low}$ monocytes represent direct precursors *in vivo* for at least some populations of bone marrow-derived short-lived macrophages and DC that differentiate in response to infection or inflammation (colored in yellow) and have the potential to migrate to secondary lymphoid organs and to regulate immune responses. Macrophages and DC that differentiate in the steady state from bone marrow derived cells are represented in green. Monocytes appear to be inefficient at generating $CD8\alpha^{+}$ and $CD8\alpha^{-}$ spleen DC subsets.

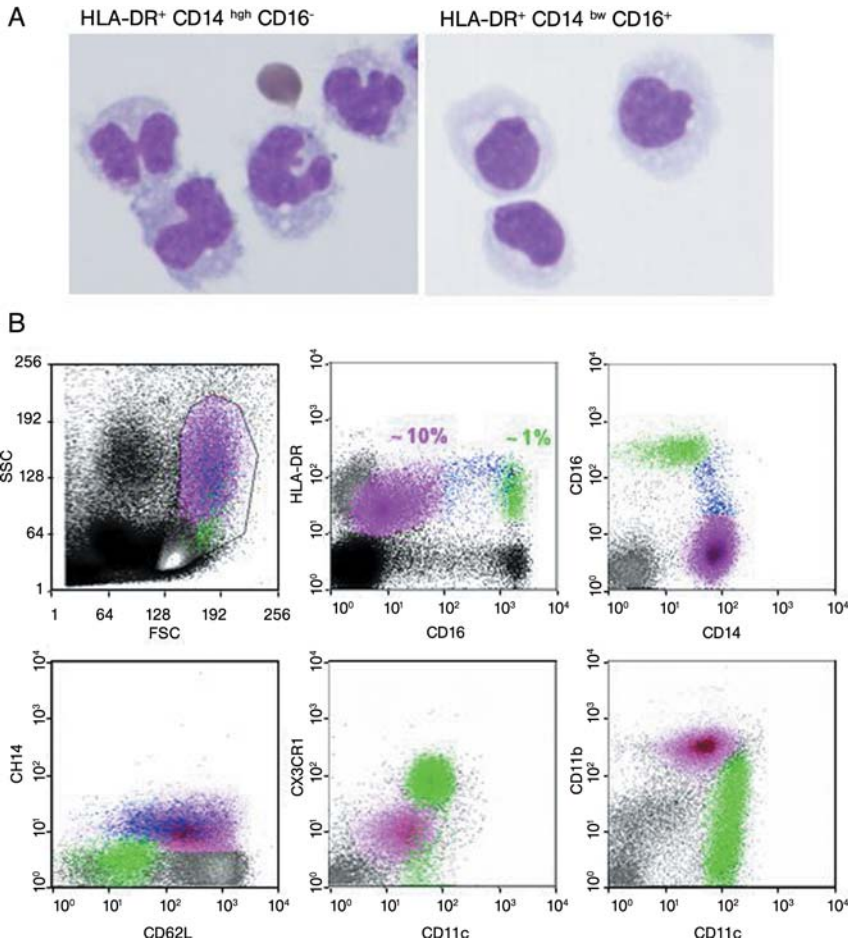


Fig. 14.2 Morphology and phenotype of human monocyte subsets. A. May-grunwald-Giemsa staining of sorted HLA-DR$^+$ CD14$^+$ CD16$^-$ monocytes (left panel), and HLA-DR$^+$ CD14$^-$ CD16$^+$ monocytes (right panel). B. Size, granularity, and surface phenotype of HLADR$^+$ CD14$^+$ CD16$^-$ monocytes (gated in pink color), HLA-DR$^+$ CD14$^-$ CD16$^+$ monocytes (gated I, green color), and of the HLA-DR$^+$ CD14$^+$ CD16$^+$ population (gated in blue). CD14$^-$ CD16$^+$ and CD14$^+$ CD16$^+$ monocytes exhibit striking differences in the shape and size of their cytoplasm and nuclei, and of their phenotype. Note that the HLA-DR$^+$ CD14$^+$ CD16$^+$ cells have FSC/SSC characteristics and phenotype similar to that of CD14$^+$ CD16$^-$ monocytes.

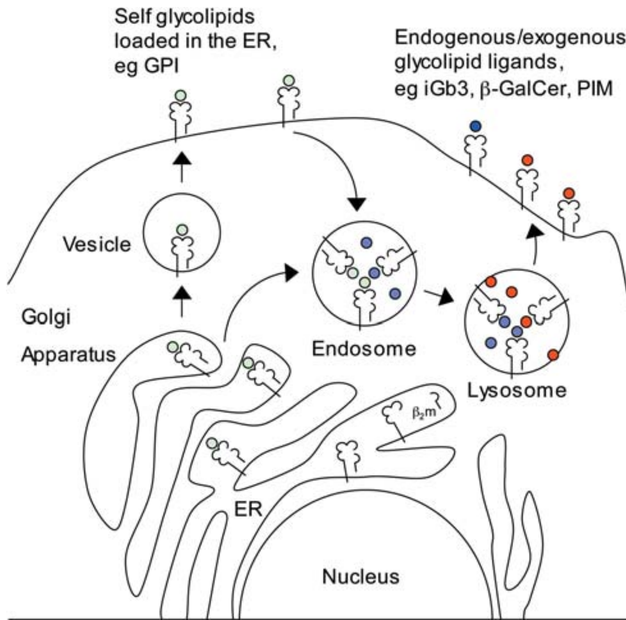


Fig. 17.1 Intracellular trafficking pathway of newly synthesized CD1d molecules. The CD1d heavy chain is folded and assembled in the endoplasmic reticulum (ER), and associates with a β_2m molecule. A self-glycolipid (indicated in green), such as GPI, binds to the CD1d molecule in the ER. The glycolipid loaded CD1d molecule is processed along the secretory pathway to the plasma membrane. A proportion of CD1d molecules associate with MHC class II/Ii chain complexes and are directed to endosomal compartments. CD1d molecules located on the plasma membrane are internalized, traffic through the late endosome/lysosome compartments, due to the internalization sequence within the cytoplasmic tail of CD1d, and are recycled back to the plasma membrane. In the endosome/lysosome compartment, CD1d exchanges the lipid it bound in the ER for a foreign glycolipid (indicated in purple) such as PIM from bacteria, encountered as free soluble molecules in the endosome, or another self-glycolipid, potentially the cellular derived iGb3 (indicated in red), encountered in the lysosome as a degradation product.

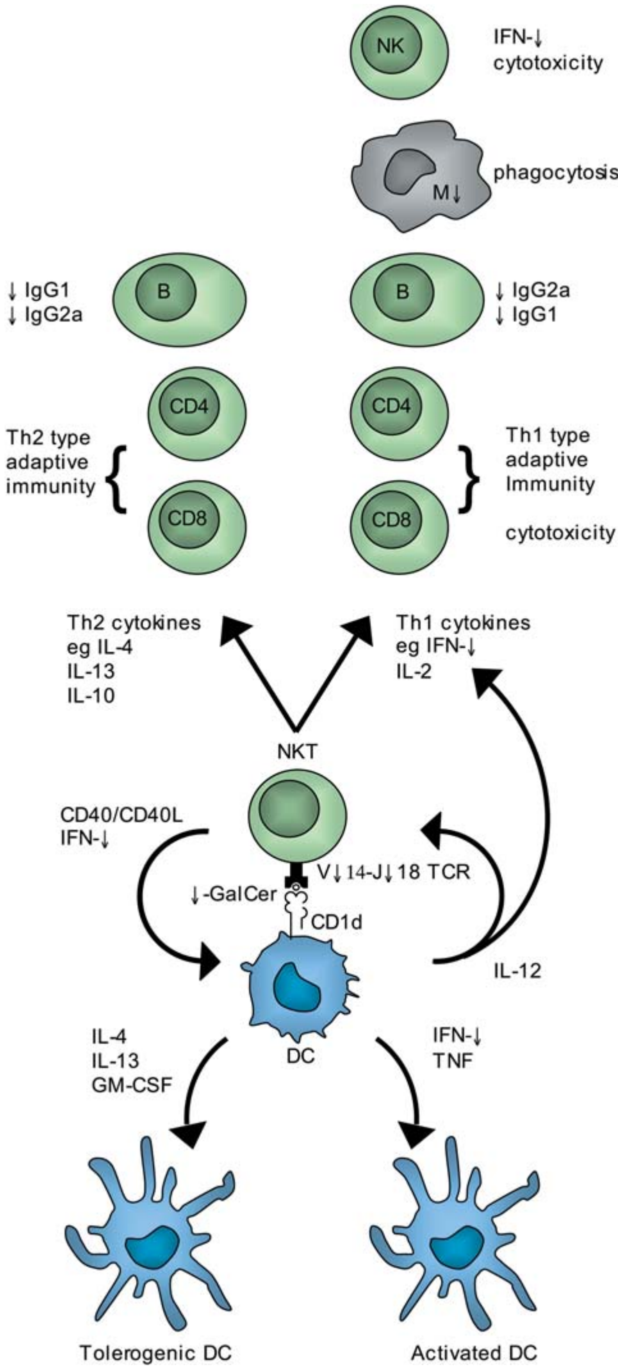


Fig. 17.2 Immunomodulatory functions of activated NKT cells, following recognition of α-GalCer presented by DC. The foreign glycolipid α-GalCer is taken up by DC and presented to the NKT cells. NKT cells receiving an activation signal via TCR ligation will rapidly release predominantly Th1 and Th2 type cytokines. DC receive maturation signals from the activated NKT, including CD40L upregulated on the NKT cell and IFN-γ, resulting in the release of IL-12 by the DC, which principally feeds back to NKT cells to induce a second burst of IFN-γ. Cytokines released by NKT cells (eg. IFN-γ, IL-4, granulocyte colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF), IL-2, IL-10, IL-13) indirectly modulate cellular components of the innate and adaptive immune system, including NK cells, macrophages, $CD8^+$ and $CD4^+$ T cells, and B cells. Immature DC can mature into activated DC or into tolerogenic DC.

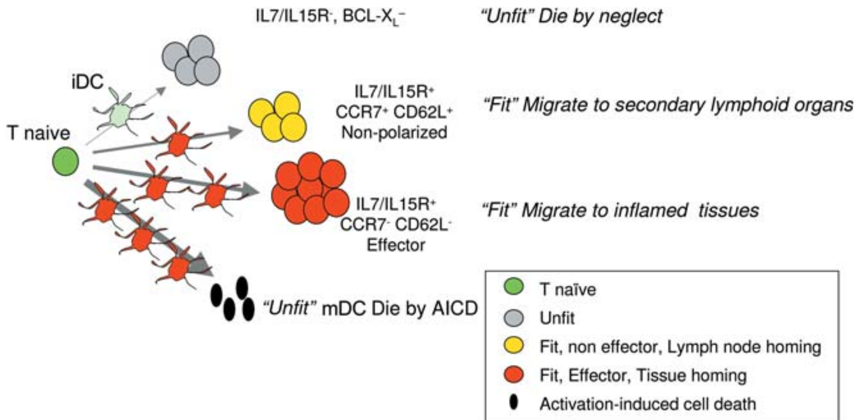


Fig. 18.1 The strength of stimulation (SoS) model of T-cell activation and differentiation. Naïve T cells interacting with antigen-presenting DC receive different SoS (depicted as the length and thickness of the arrow) depending on the DC maturation state the concentration of peptide–MHC and co-stimulatory molecules on DCs and on the duration of T–DC interaction. Short interactions with immature DC (low SoS) are sufficient to induce proliferation, but the proliferating T cells are unfit and do not survive. At higher levels of SoS, proliferating T cells become fit and either remain non polarized of differentiate to effectors under the aegis of polarizing cytokines. Excessive SoS leads to activation induced cell death. iDC, immature DC; mDC, mature DC.

Color Plates

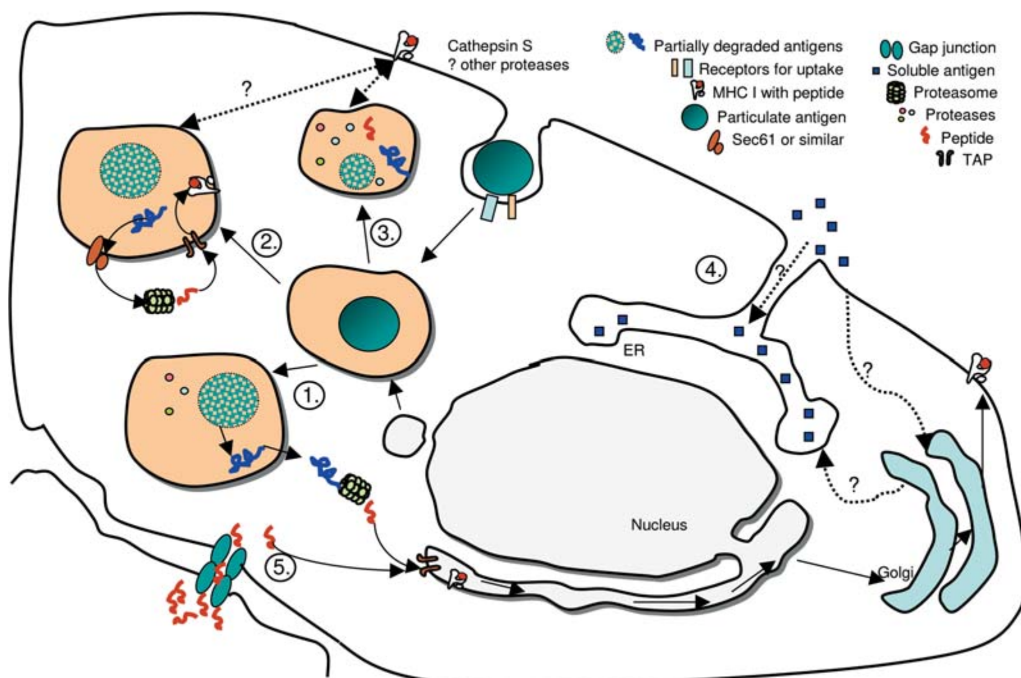


Fig. 22.1 Mechanisms of crosspresentation. Particulate antigens enter the antigen presenting cells most efficiently by receptor mediated endocytosis. Endosomes containing the ingested antigen then acquire selected endoplasmatic reticulum (ER)-derived elements and, after initial processing within the endosome, the antigens exit the late endosome and enter the cytoplasm. They then enter the classical pathway and are processed in a similar manner as endogenous antigens (1). Alternatively, the antigens may remain in the endosomes that acquire selected characteristics of ER and are fully processed in close viscinity of the phago-endosome. They re-enter the phago-endosomes and are there loaded onto MHC class I molecules (2). Whereas in the former cases the antigen processing involves transporter of antigen processing (TAP), this is not always required. Albeit less efficiently, the vacuolar processing pathway (3) may rely on vacuolar proteases, especially cathepsin S, to generate peptides that are loaded onto MHC class I molecules within the vacuole. A special pathway may be in place for soluble antigens (4) which by yet unidentified mechanisms directly access the ER. Gap junctions may permit the transfer of already processed peptides from donor to acceptor cells (5).

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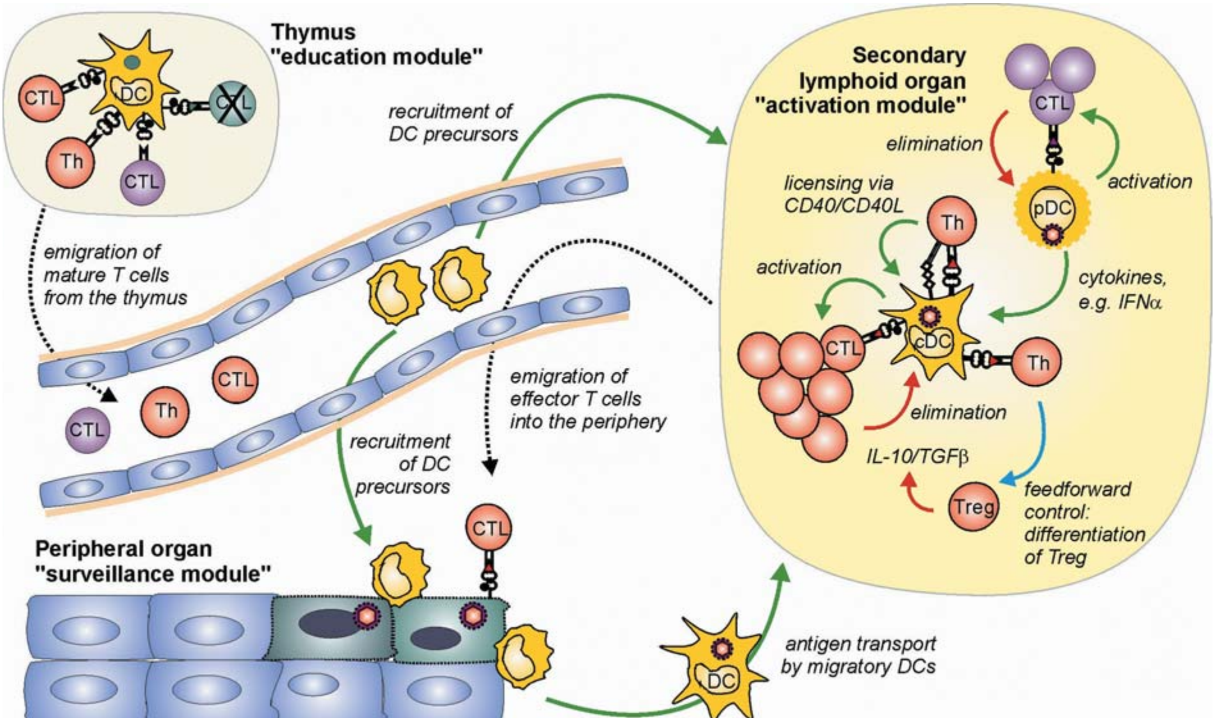

Fig. 23.1 Systems biologist's view of DC–CTL interactions. *Modularity* in the complex system of (direct and indirect) DC–CTL interactions is represented by different organizational modules: thymus ("educational module"), secondary lymphoid organs ("activation module"), and peripheral nonlymphoid organs ("surveillance module"). Different DC subsets, e.g. conventional DCs (cDC) versus plasmacytoid DCs (pDC) provide heterogenous *redundancy* and ensure homogenous outcome, that is, activation of CTL and secretion of stimulating cytokines. Positive *feedback* mechanisms (e.g. chemokine-driven recruitment of DC precursors into peripheral organs or secondary lymphoid organs, or CD40-mediated licensing of DCs) are indicated by green arrows. Negative feedback control loops such as elimination of DCs by effector CTL are depicted in red. Generation of regulatory Th cells (Treg) can be seen as feedforward control (blue arrow).

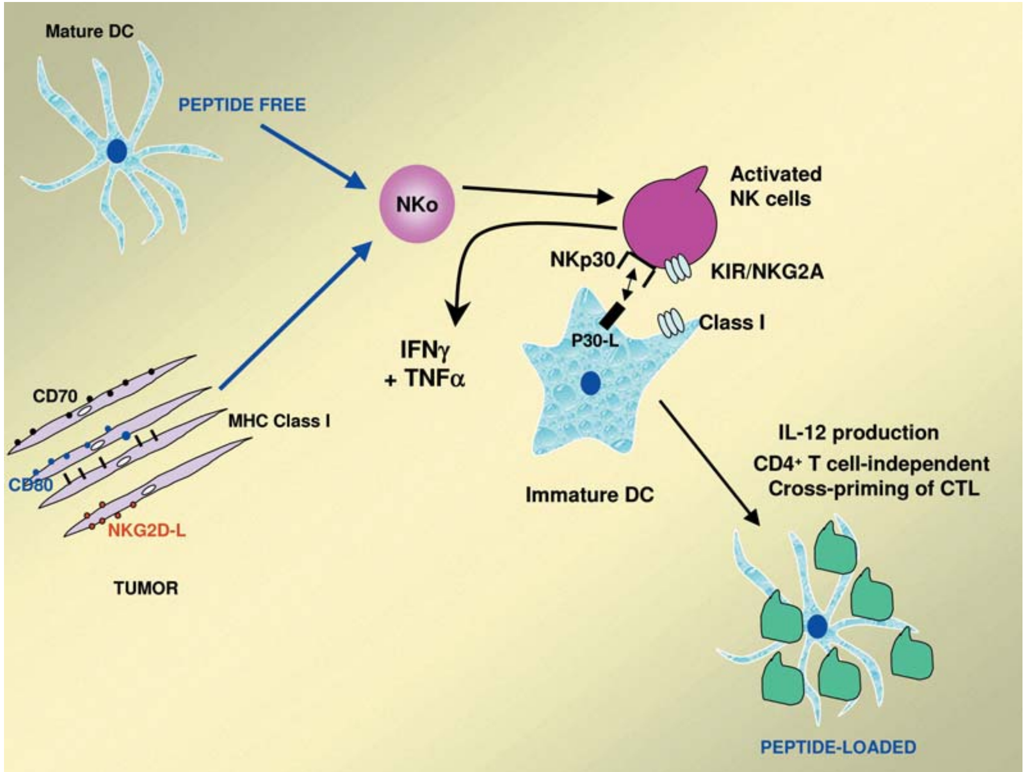

Fig. 24.1 A DC/NK/DC cross-talk leading to efficient cross-presentation of tumor antigens. Tumor cells over-expressing NKG2D ligands or CD70 or CD80 might directly trigger NK-cell activation. Alternatively, exogenous LPS-activated mature DC can turn on NK cells *in vivo*. DC-activated NK cells produce IFNγ which in turn, promotes endogenous DC activation and production of IL-12 and elicitation of CTL cross priming in the absence of $CD4^+$ T-cell help [43]. In the human setting, DC-activated NK cells or IL-2 activated NK cells will induce DC maturation through engagement of NKp30, in a TNF-α dependent-manner.

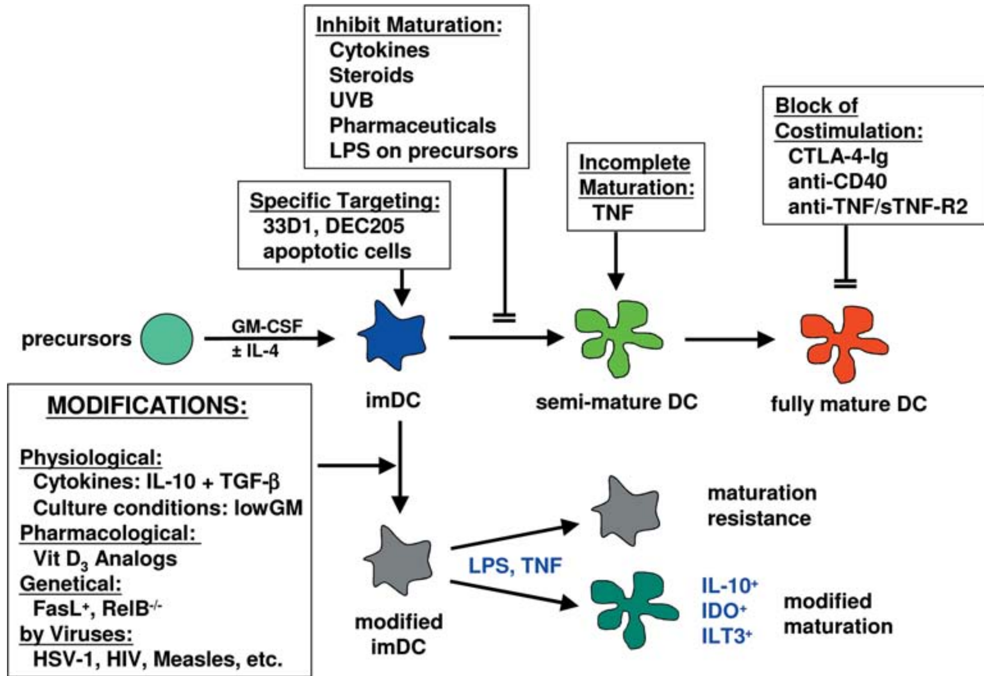

Fig. 26.1 Methods of *in vitro* generation or *in vivo* targeting of tolerogenic DC. Many methods of generating tolerogenic DC are focused around immature DC. They can be targeted specifically *in vivo* by delivery of apoptotic material or targeting to specific surface receptors. Spontaneous maturation of *in vitro* generated DC can be achieved by addition of maturation inhibitors. Inhibition of *in vivo* maturation is attempted by inhibiting co-stimulation of already matured DC. Incompletely matured DC (semi-mature DC) can still be tolerogenic. Due to the problem of maintaining stable immature DC, DC modifications are desired that result in maturation-resistant immature DC or modified/alternatively matured DC expressing inhibitory surface receptors (IDO, ILT-3) or cytokines (IL-10).

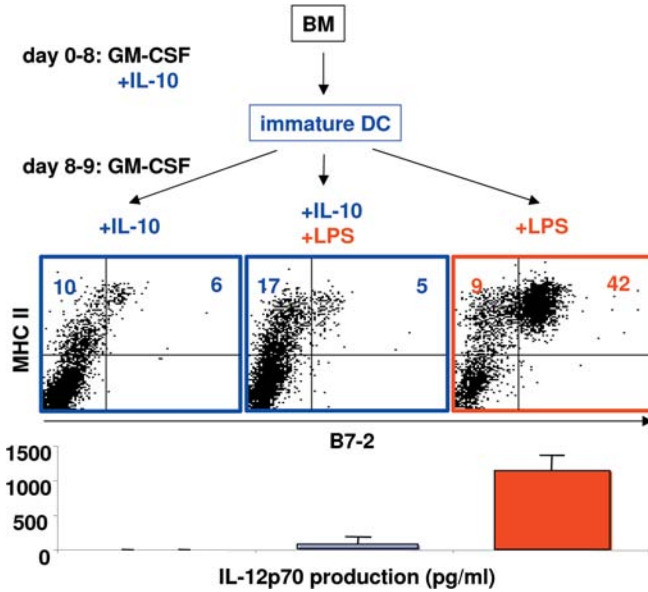

Fig. 26.3 Importance of maturation resistance by immature DC for their tolerogenicity. BM-DC were cultured for 8 days with GM-CSF (200 U ml^{-1}) plus IL-10 (10 ng ml^{-1}) to inhibit spontaneous maturation. Then the cells were washed and replated with GM-CSF and IL-10, or IL-10 plus LPS or LPS for another 24 h. FACS analysis was performed with the cells for surface MHC II (M5/114-PE) and B7-2 (FITC) and the IL-12p70 production measured by ELISA. The data show that in the absence of the inhibitory IL-10 signal, the immature DC rapidly mature on LPS.

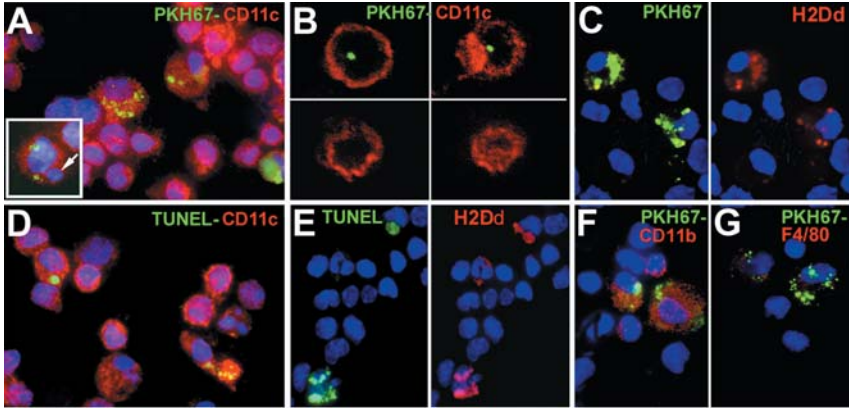

Fig. 29.3 Entrapment of apoptotic cells by splenic DC *in vivo*. Internalization of apoptotic cells by splenic DC was analyzed in cytospins of immunobead-sorted DC 1 h after injection of PKH67-labeled (green) apoptotic (BALB/c) splenocytes in (B10) mie. (A) CD11c^{+} DC with apoptotic cell fragments (green) and with DAPI^{+} intracytoplasmic inclusions, -likely DNA from ingested apoptotic cells (in blue indicated by arrow in inset). (B) Serial sections analyzed by confocal microscopy confirmed the intracellular localization of PKH67-labeled fragments in splenic CD11c^{+} DC. (C) The donor origin (BALB/c) of the intracytoplasmic inclusions in (B10) DC was confirmed by H2D^{d} expression (in red) in PKH67-labeled (green) fragments. (D & E) FITC-TUNEL staining in combination with Cy3-anti-CD11c or Cy3-anti-H2D^{d} confirmed the presence of donor (BALB/c)-derived apoptotic cells within (B10) DC. (F & G) One h after i.v. injection of apoptotic cells, DC that internalized apoptotic cells expressed CD11b^{hi} and F4/80$^{lo/-}$. Nuclei were counterstained with DAPI (1000x). Reproduced with permission from *Blood* **2003**. 101: 611–620 (A.E. Morelli et al.).

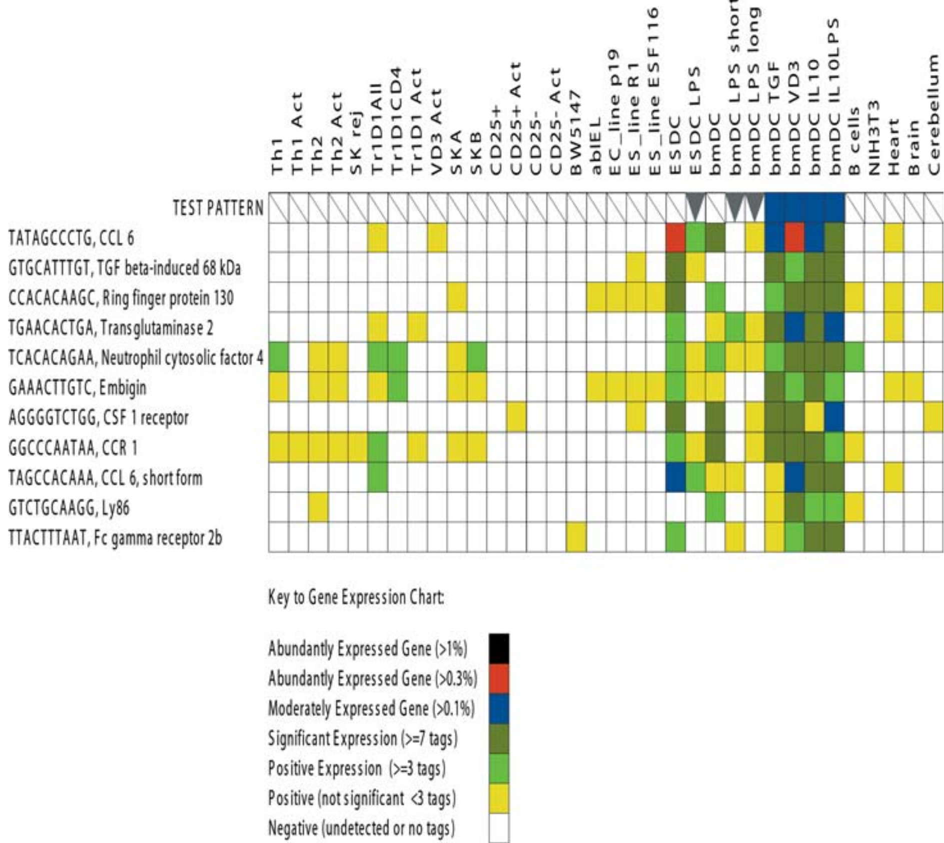

Fig. 30.6 SAGE tags associated with modulated DCs. Using SAGEClus software, tags associated with the pharmacologically-modulated DC populations have been selected based on the relatedness of their expression profiles to the idealized test pattern indicated in the top row of the clustergram. A blue square in the test pattern indicates moderate tag representation in that library, as indicated in the expression key, while an inverted grey triangle indicates no tag representation and a diagonal line no preference. Details of the comparator libraries indicated can be found in [86].

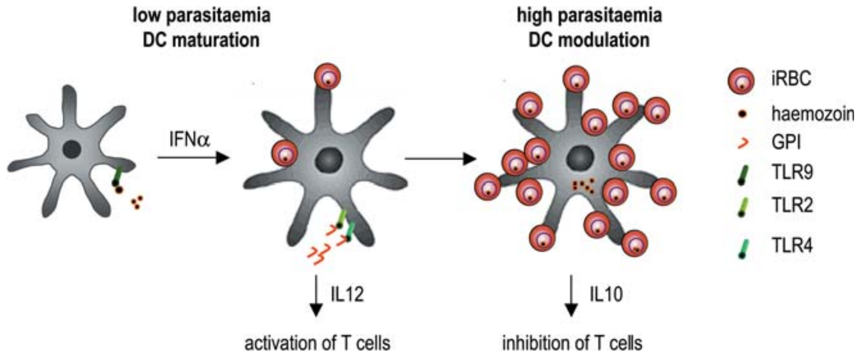

Fig. 31.1 Simplified diagram on the effect of parasitemia on DC function. Early on during infection, engagement of TLR9 by hemozoin results induces plasmacytoid DC to secrete IFNα and engagement of TLR2 and TLR4 by GPI induces myeloid DC to secrete IL12. With increasing parasitemia, more and more myeloid DC in the spleen might be modulated either directly through interaction with iRBC or through ingestion of increasing amounts of hemozoin and secrete IL10.

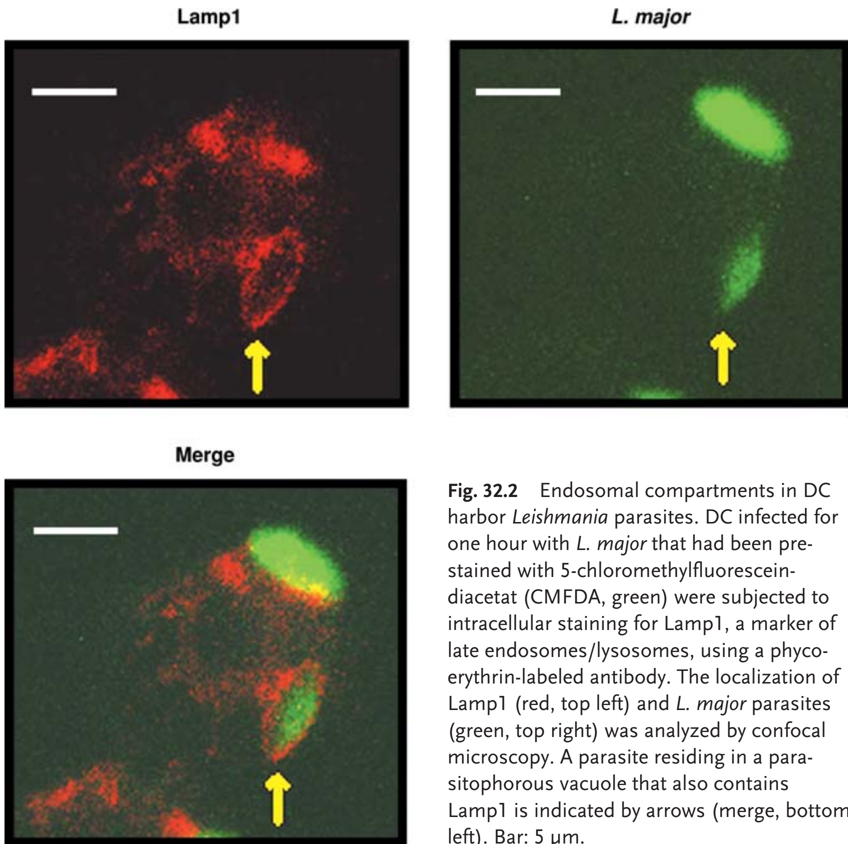

Fig. 32.2 Endosomal compartments in DC harbor *Leishmania* parasites. DC infected for one hour with *L. major* that had been pre-stained with 5-chloromethylfluorescein-diacetat (CMFDA, green) were subjected to intracellular staining for Lamp1, a marker of late endosomes/lysosomes, using a phycoerythrin-labeled antibody. The localization of Lamp1 (red, top left) and *L. major* parasites (green, top right) was analyzed by confocal microscopy. A parasite residing in a parasitophorous vacuole that also contains Lamp1 is indicated by arrows (merge, bottom left). Bar: 5 μm.

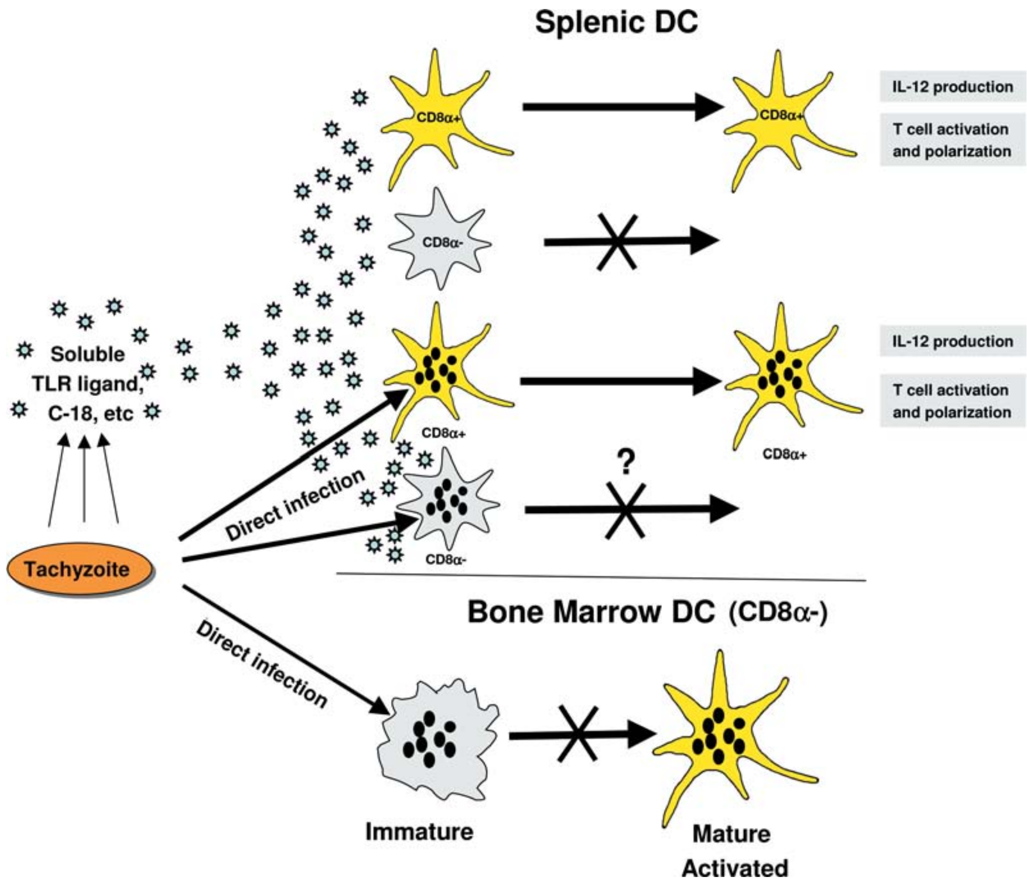

Fig. 33.1 Encounter of DC subsets with *T. gondii* leads to distinct outcomes. CD8α^+ but not CD8α^- DC in spleen and other tissues respond to soluble products released by tachyzoites and present in STAg. Whether intracellular tachyzoites (as opposed to soluble parasite products) can trigger responses in CD8α^- DC, particularly after extended incubation, remains to be determined. While direct infection does not appear to influence the activation status of splenic DC, bone-marrow-derived DC not only fail to respond to soluble tachyzoite Ag but when infected with the parasite display a block in maturation and response to exogenous stimulation with LPS or CD40L [14].

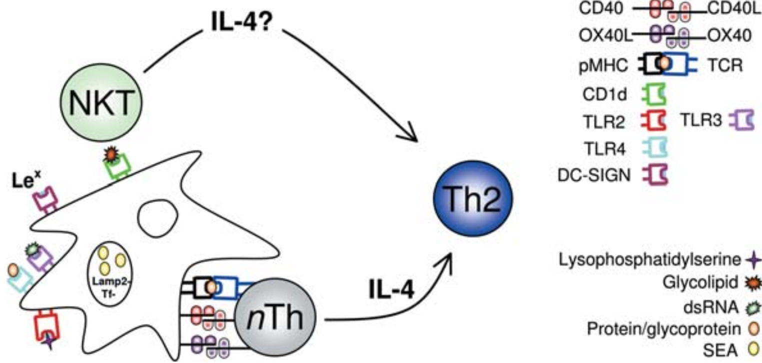

Fig. 34.2 Th2 induction by DC in response to SEA. It is likely that multiple components of SEA bind to a range of pattern recognition receptors on DC, including C-type lectins [19] and TLRs [22, 25, 27]. Although several TLRs have been implicated in this process, MyD88-deficient mice are still capable of making Th2 responses to SEA [66], suggesting either a degree of redundancy in the system or, more likely, that MyD88-independent TLR-initiated signaling is important. Once internalized SEA is distributed to $Lamp2^-$, $Transferrin^-$ vesicular compartments within the DC [18]. CD40 expression is critical for DC induction of Th2 response to SEA [46], suggesting downstream involvement of CD40-mediated activation events in this process, with OX-40L being one potential candidate that could fulfill this function. IL-4-deficient DC show no deficiency in Th2 induction to SEA [41], although IL-4 from a source other than the initiating DC is paramount to sustain the developing CD4 T-cell response. NKT cells may provide one source of such IL-4, as CD1d-deficient DC display impaired Th2-induction abilities to SEA [35].

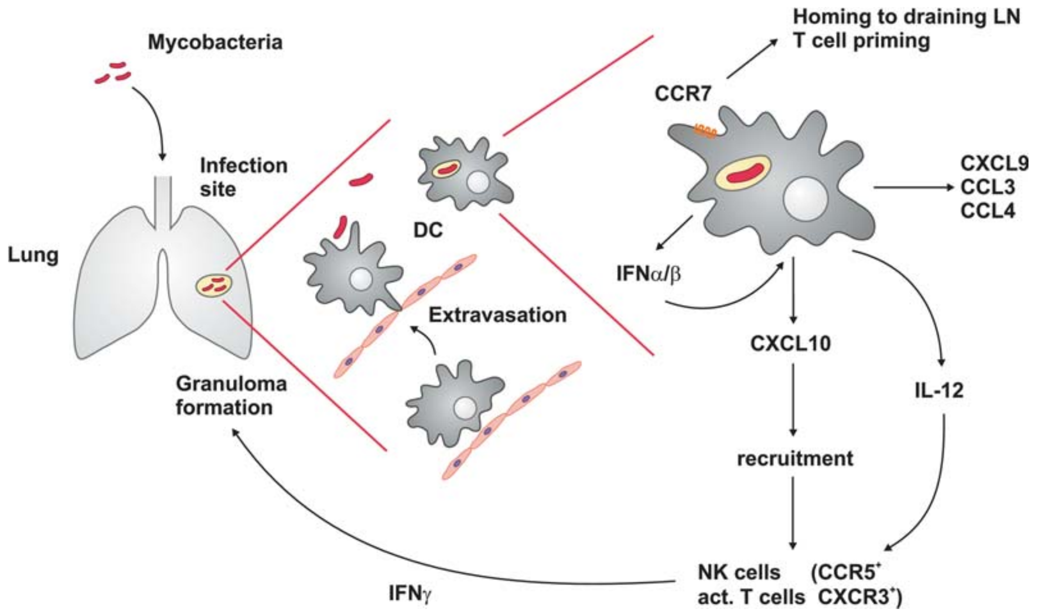

Fig. 36.1 Role of DC in tuberculosis. Inhalation of mycobacteria by aerosol leads to the infection of macrophages and DC in the lung. Infection activates DC to express CCR7 and to subsequently migrate to draining lymph nodes for T-cell priming. Concurrent release of type I IFN-α/β starts an autocrine activation loop initiating chemokine secretion (CXCL10, 9, CCL3, 4) to recruit activated T cells and NK cells to the site of infection. Onset of a protective T-cell response with IFNγ and TNFα as essential cytokines leads to macrophage/DC activation and granuloma formation to restrict spread of mycobacteria.

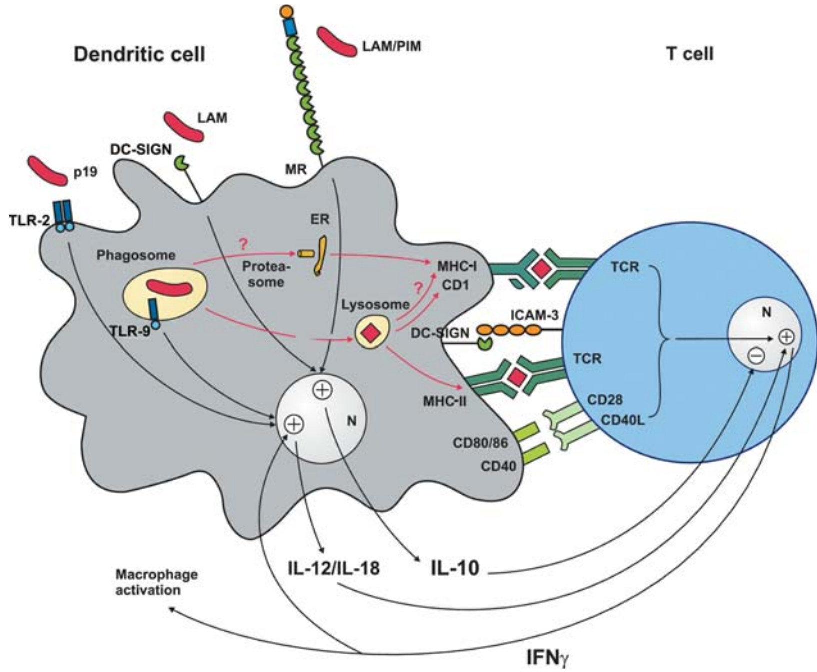

Fig. 36.2 The DC as immune-modulating APC. Mycobacteria and their pathogen associate molecular patterns (PAMP) such as lipoarabinomannan (LAM), the 19-kDa lipoprotein (p19) and low-methylated bacterial DNA (CpG) bind various pattern recognition receptors (PRR) on DC. Engagement of DC-SIGN and the mannose receptor (MR) induce release of the anti-inflammatory/immunosuppressive cytokine IL-10. In contrast, ligands for Toll-like receptors (p19 – TLR-2, CpG – TLR-9) activate DC to secrete the pro-inflammatory cytokines IL-12/IL-18. Thereby an IFNγ-dominated T-cell response (T helper type I) is initiated leading to macrophage activation and mycobactericidal effector mechanisms. DC are potent antigen-presenting cells employing MHC-I, -II, CD1 and co-stimulatory molecules (CD80, CD86, CD40) to prime mycobacterium-specific T cells. Mycobacterial antigens are delivered to the lysosomes for processing and binding to MHC-II and CD1 molecules. Loading of lipids onto CD1 molecules involves the lipid transfer proteins saposins. The pathway leading to MHC-I presentation within infected cells – whether through processing and loading within late endosomes/lysosomes, processing by proteasomes and loading in the ER, or by both – is yet unclear.

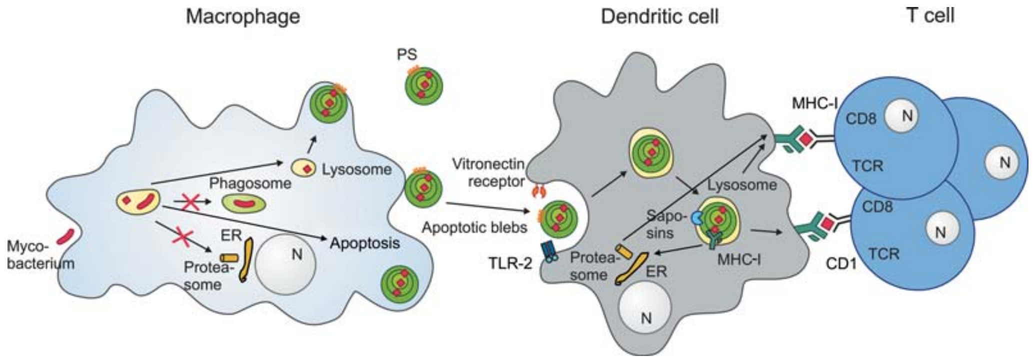

Fig. 36.3 The detour pathway of MHC-I and CD1 presentation. In macrophages, mycobacteria are segregated within phagosomes from the classical MHC class I pathway. Moreover, macrophages do not express group I CD1 molecules (CD1a, b, c). Finally, mycobacteria-infected cells loose their ability to present antigens to T cells. These hinderances to induce proper T-cell immunity are overcome by the detour pathway in tuberculosis. Infection-induced apoptosis leads to the release of phosphatidyl-serine (PS)-positive apoptotic blebs from infected cells. Thereby, mycobacterial antigens are carried to non-infected DC for presentation. Apoptotic blebs are engulfed by the vitronectin receptor (VR) and probably the PS-receptor and reach the endosomal system of the DC. DC maturation is initiated upon engagement of TLR-2 by mycobacterial PAMP (such as p19). DC subsequently prime T cells through MHC-I and CD1, but also MHC-II molecules. Processing of mycobacterial antigens is predominantly dependent on the lysosomal pathway. Saposins are involved in this process.

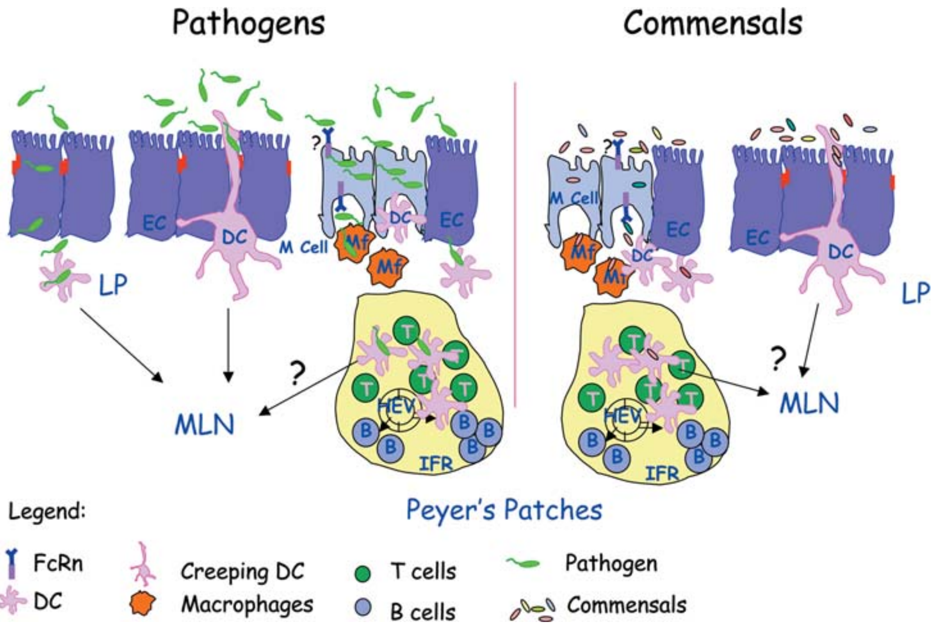

Fig. 37.1 Mechanisms of bacterial uptake. The mechanisms of bacterial entrance depend on their pathogenicity. Most of the pathogens have developed strategies to penetrate ECs or to facilitate M-cell invasion, alternatively they are captured by creeping DCs (left). Commensal bacteria can enter mucosal surfaces either through M cells or DCs (right). M cells can release their 'cargo' to underlying phagocytic cells, including DCs, that can migrate to the interfollicular region (IFR) of Peyer's Patches for T and B-cell interactions, whereas DCs that take up bacteria directly across mucosal surfaces are likely to migrate to MLN. Alternatively, PP-DCs could migrate to MLN. An alternative mechanism for antigen entry across a mucosal surface that also targets DCs and could be used for bacterial internalization is mediated by neonatal Fc receptors (FcRn) expressed by adult human (but not mouse) intestinal epithelial cells. FcRn transport directs and delivers the antigens in the form of immune complexes directly to underlying DCs. (HEV: high endothelial venules).

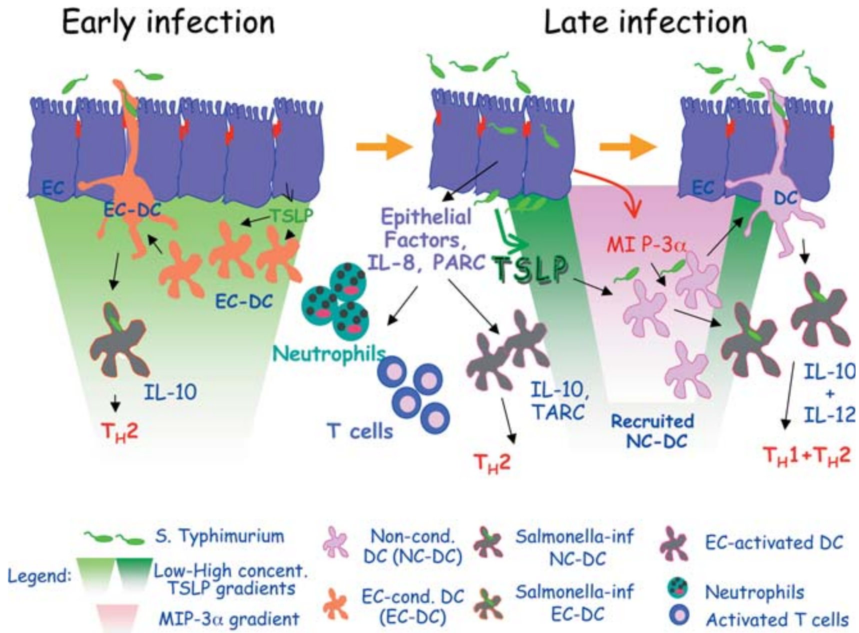

Fig. 37.2 Early *Salmonella typhimurium* infection: resident DCs are conditioned by EC-released TSLP (EC-DC). EC-DC release IL-10 after bacterial exposure and drive default T_H2 responses to *S. typhimurium*.
Late infection: since *S. typhimurium* is an invasive bacterium, it induces ECs to release pro-inflammatory chemokines like IL-8 (CXCL-8) and PARC (CCL-18), which attract neutrophils, granulocytes and activated T cells that generate an inflamed site. The binding of *Salmonella* to the basolateral membrane of ECs induces the upregulation of TSLP. TSLP at this concentration drives T_H1 rather than T_H2 promoting DCs in response to bacteria. Unidentified EC-derived factors can also activate 'bystander' DCs that have not been in contact directly with the bacteria. DCs activated in this way release IL-10 and TARC (CCL-17) but not IL-12, thus driving and recruiting T_H2 T cells. *Salmonella* also induces the release of MIP-3α (CCL-20) that recruits CCR6-expressing immature DCs.
Most likely, recruited DCs are not subjected to EC-conditioning, rather they could find increased TSLP concentrations in the infected site. Newly recruited DCs (NC-DC) can either creep between ECs to take up bacteria or they can phagocytose bacteria that have breached across the epithelial barrier and release both IL-10 and IL-12, thus promoting T_H1 and T_H2 responses. This allows the establishment of protective anti-*Salmonella* responses.

Color Plates

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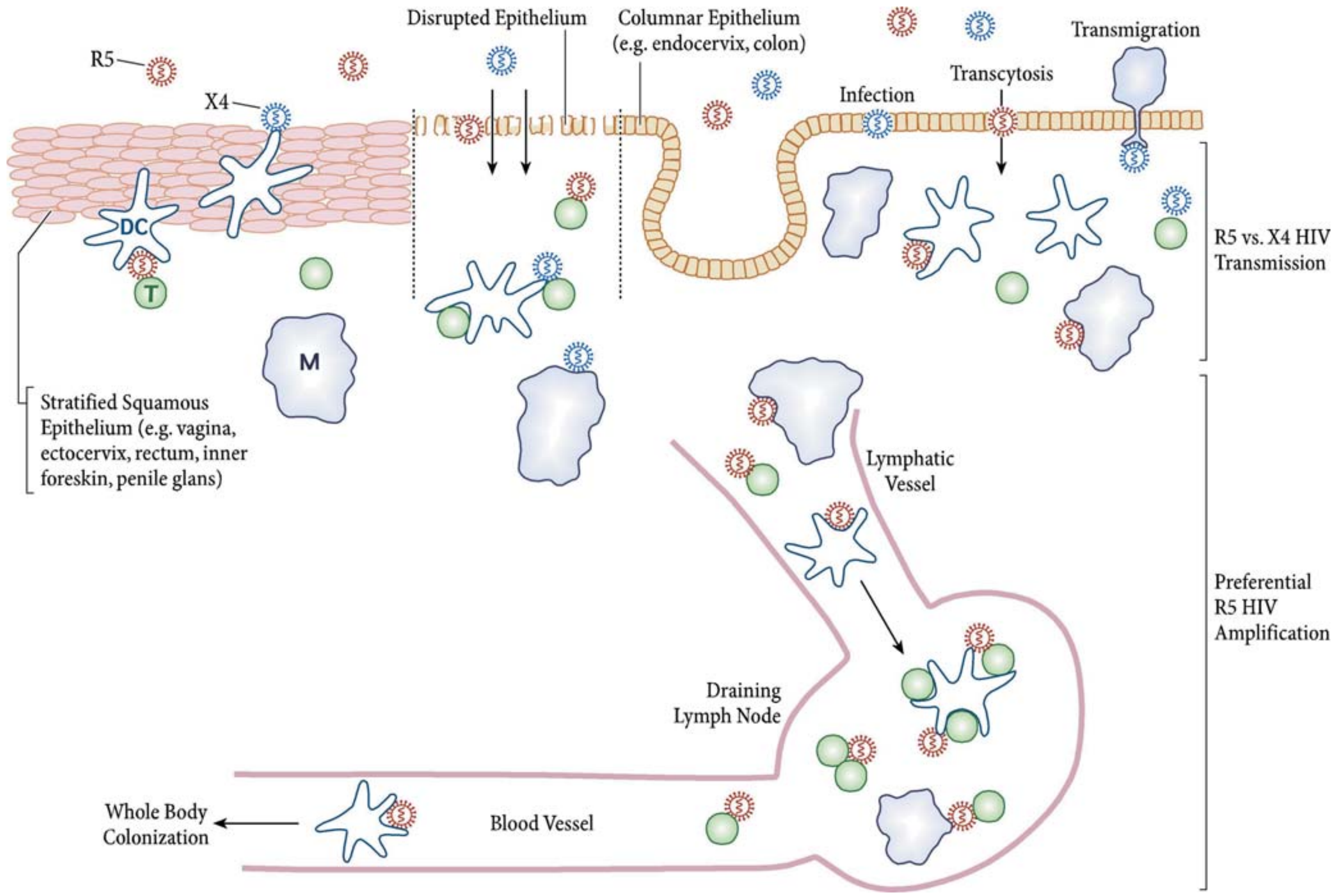
R5
X4
DC
T
M
Disrupted Epithelium
Columnar Epithelium
(e.g. endocervix, colon)
Infection
Transcytosis
Transmigration
R5 vs. X4 HIV
Transmission
Stratified Squamous
Epithelium (e.g. vagina,
ectocervix, rectum, inner
foreskin, penile glans)
Lymphatic
Vessel
Preferential
R5 HIV
Amplification
Draining
Lymph Node
Whole Body
Colonization
Blood Vessel

Fig. 38.1 Potential cellular targets during mucosal transmission of HIV. At mucosal surfaces, the epithelium represents the first line of defense for the body. HIV needs to cross this protective layer to access its target cells and initialize an infection. This event is dependent on the thickness and integrity of the tissue. Faced with a disrupted epithelium (breaks due to physical injury or infectious lesions caused by other pathogens) the virus has direct access to the subepithelial resident cells constituting a mixture of DCs (DC), T cells (T), and macrophages (M), which is a favorable environment for local viral amplification. In the presence of an epithelial monolayer, the virus has several possibilities to reach the subepithelial compartment. HIV can either infect, transcytose, or pass between the epithelial cells. Similar events may happen in the presence of a multilayered epithelium, but in that case the probability for the virus to cross the entire layer is reduced. LCs located in the epithelial cell network may "trap" the virus using their extensions and conduct it directly to the subepithelial compartment. Once bearing the virus (as infected cells or cells with internalized virus; Fig. 38.3), DCs can migrate to the afferent lymph nodes where the virus is transmitted to the T cells, facilitating systemic virus dissemination. While both R5 and X4 viruses possibly cross the barrier, there appears to be preferential amplification and dissemination of R5 viruses.

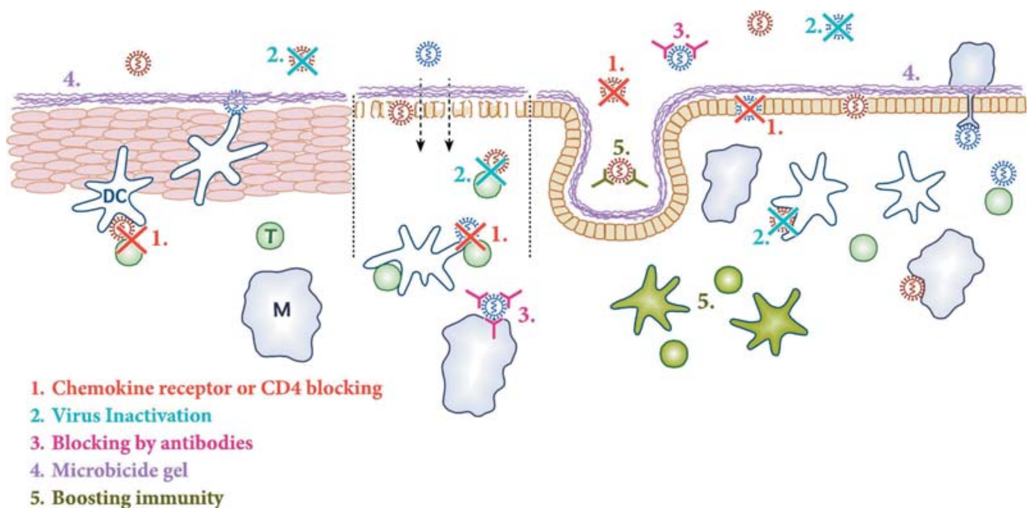

Fig. 38.4 Antiviral actions at the surfaces and in the tissues. Multiple strategies are being explored to block the virus entry and spread. One approach consists of blocking infection using CD4 or CCR antagonists, which bind to the permissive cells. Preventing binding of envelope to host cells can be achieved using neutralizing anti-envelope antibodies or agents like CNV. Another type of microbicide would prevent virus entry by nonspecific saturation of the virus-cell interactions (e.g. sulfated polymers). Inclusion of antiviral drugs that prevent infection by targeting infectious virus would improve these strategies that preferentially block the initial virus attachment. Boosting the host immunity by vaccination will lead to the emergence (and/or expansion) of HIV-specific immune cells at the mucosal sites that can directly target the virus (e.g. antibodies) and virus-infected cells (e.g. cytotoxic T cells). Boosted innate immune responses could also limit incoming infections as well as reduce the spread of infectious virus being shed.

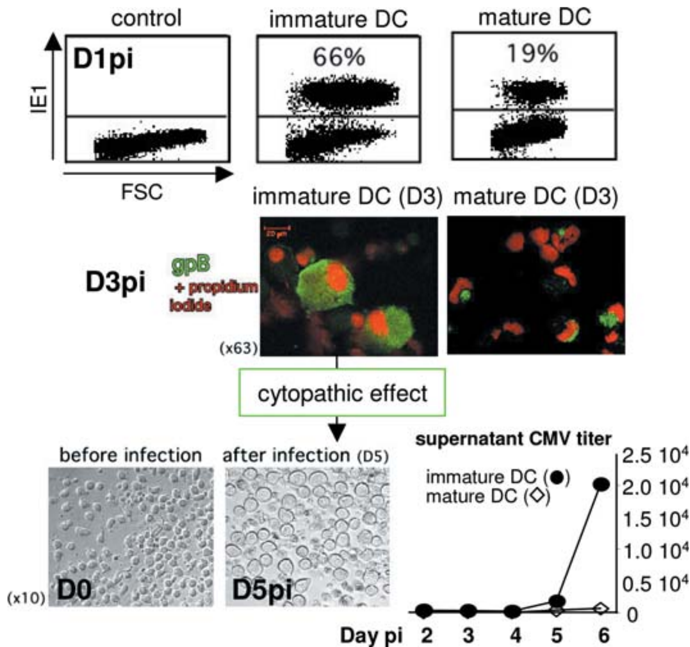

Fig. 39.2 DCs are permissive for CMV infection. Immature and mature monocyte-derived DCs (moDCs) express immediate-early-1 (IE1) antigen at day 1 post infection (D1pi). IE1 expression is always greater in immature than mature moDCs, however, at the same multiplicity of infection (top panel). A late replication step is also achieved as proven by the expression of glycoprotein B (FITC) with propidium iodide for nuclear staining (middle panel), with different patterns between immature and mature moDCs. Indeed immature moDCs, but not mature moDCs, display the typical cytopathic effect (D5pi versus D0 in the photomicrographs, bottom panel) and release infectious viral particles during late replication steps (HCMV supernatant titer, far right, bottom panel). (These data were originally published in Senechal et al., Infection of mature monocyte-derived dendritic cells with human cytomegalovirus inhibits stimulation of T-cell proliferation via the release of soluble CD83. Blood. **2004**, 103: 4207-4215. © 2004 The American Society of Hematology.)

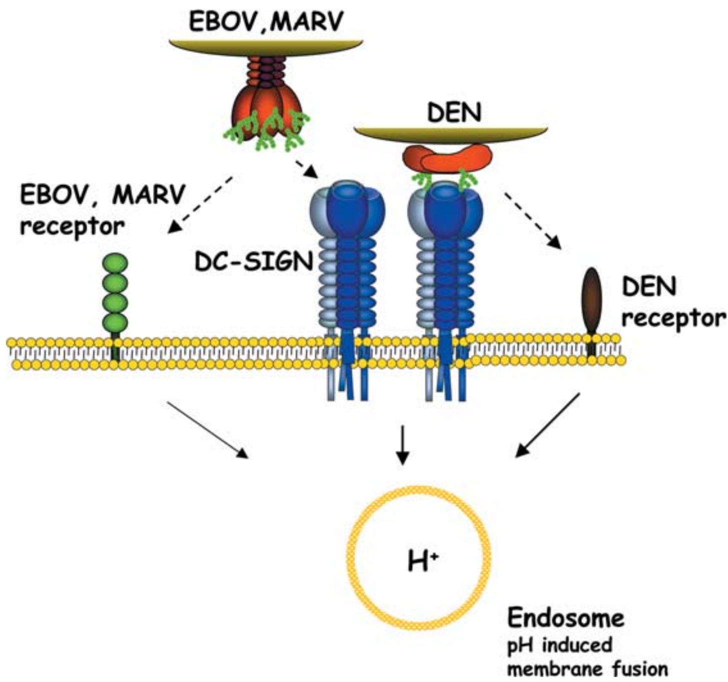

Fig. 40.2 Filovirus and DEN interactions with DCs. DEN engages DC-SIGN for entry into DCs. DC-SIGN might not facilitate infectious entry but augment DEN infection via a so far unidentified receptor. DC-SIGN expression on cell lines augments EBOV, MARV infection, however, it is unclear if DC-SIGN binding contributes to infection of DCs. Upon EBOV, MARV and DEN uptake into DCs via DC-SIGN and/or cellular receptors, virions are transported into endosomal vesicles, in which protonation triggers glycoprotein mediated membrane fusion.

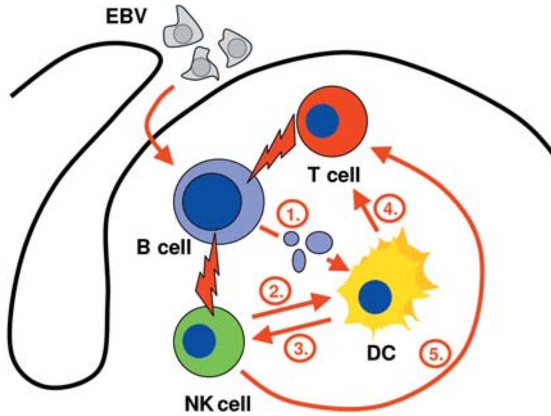

Fig. 43.1 Dendritic cells (DCs) coordinate the immune response to Epstein–Barr virus (EBV) in the tonsils. After EBV enters tonsils probably via tonsillar crypts and infects B cells, DCs sense the infection either directly through fragments of EBV-infected B cells or viral particles (1) or are matured via other innate lymphocytes like for example NK cells (2), which detect B-cell transformation by EBV. DCs then activate NK cells as a first line of defense (3) before priming EBV-specific T-cell responses (4). NK cells assist DCs in the polarization of EBV-specific T-cell responses towards protective Th1 immunity (5).

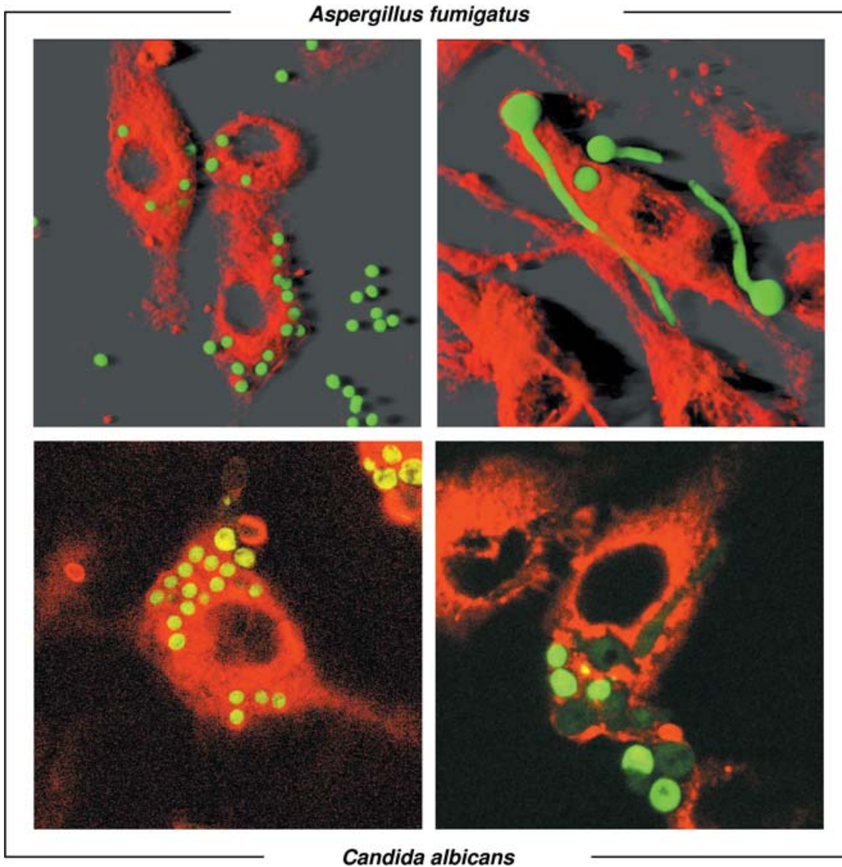

Fig. 44.2 Murine dendritic cells phagocytose different fungal morphotypes. The pictures illustrate a three-dimensional reconstruction of confocal microscopy sections of fetal skin-derived dendritic cells phagocytosing *Aspergillus fumigatus* conidia and hyphae (top panel) or *Candida albicans* yeasts and hyphae (lower panel). Reconstruction was performed with the isosurface module of Imaris software (Bitplane) on a SGI octane workstation. Cells were stained with the DiQ vital stain. GFP-expressing *Aspergillus* was kindly provided by Margo M Moore (British Columbia Canada) and GFP-expressing *Candida* by Joachim Morschhäuser (Würzburg Germany).

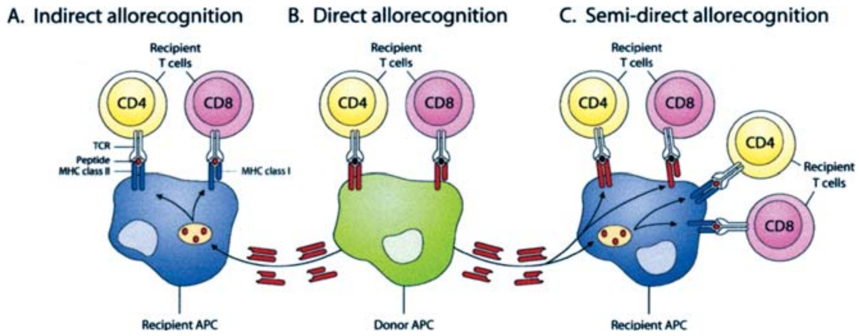

Fig. 46.1 Pathways of allorecognition. In the indirect pathway (A), alloantigens are either shed from donor cell surface or are taken up as dying or apoptotic allogeneic cells by recipient antigen-presenting cells (APC). Peptides (red circle) derived from allogeneic MHC class I or class II molecules are re-presented on the self-MHC class II or class I molecules of recipient APC. In the direct pathway (B), recipient $CD4^+$ and $CD8^+$ T cells recognize respectively donor intact allogeneic MHC class II or class I molecules bound to peptides at the surface of donor APC. In the semi-direct pathway (C), recipient APC would present to recipient T cells intact MHC class I and class II molecules transferred from donor APC simultaneously with donor MHC peptides to $CD4^+$ and $CD8^+$ T cells with indirect allospecificity.

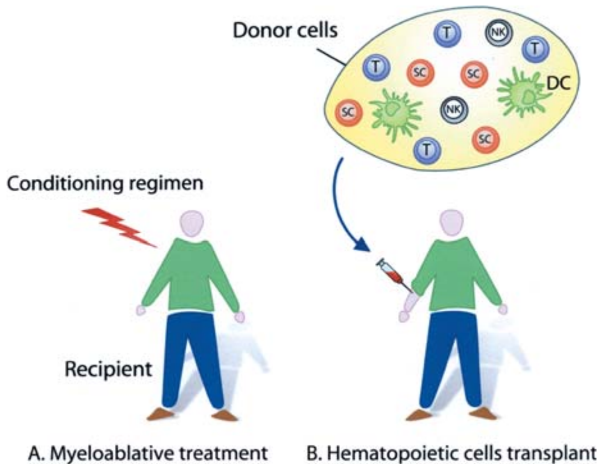

Fig. 46.2 Hematopoietic cells transplantation. Haematopoietic cell transplantation procedures consist of a preparatory conditioning regimen followed by the transplantation itself. (A) The cancer patient is treated with chemotherapy and irradiation to kill tumor cells and ablate the bone marrow. (B) The patient is infused with a preparation of donor cells that contains haematopoietic stem cells (SC). The transplanted inoculum contains mature blood cells of donor origin, including T cells, natural killer (NK) cells and dendritic cells (DC). After transplantation, the progeny of the stem cells repopulate the haematopoietic system of the patient.

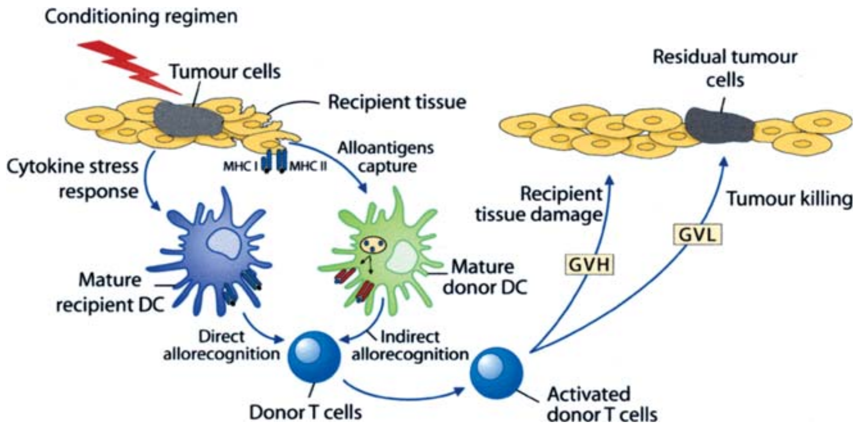

Fig. 46.3 Graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) reaction. GVHD disease is caused by mature T cells in the hematopoietic cell graft attacking tissues of the recipient that have been stressed by the myeloablative conditioning regimen. On the one hand, the cytokine stress response may lead to recipient DC maturation that would directly activate alloreactive donor T cells. On the other hand, the conditioning regimen causing tissue damages would favor the indirect presentation of recipient alloantigens by donor DC to donor T cells. Activated alloreactive donor T cells can also mediate GVL activity by recognition of alloantigens on recipient tumor cells.

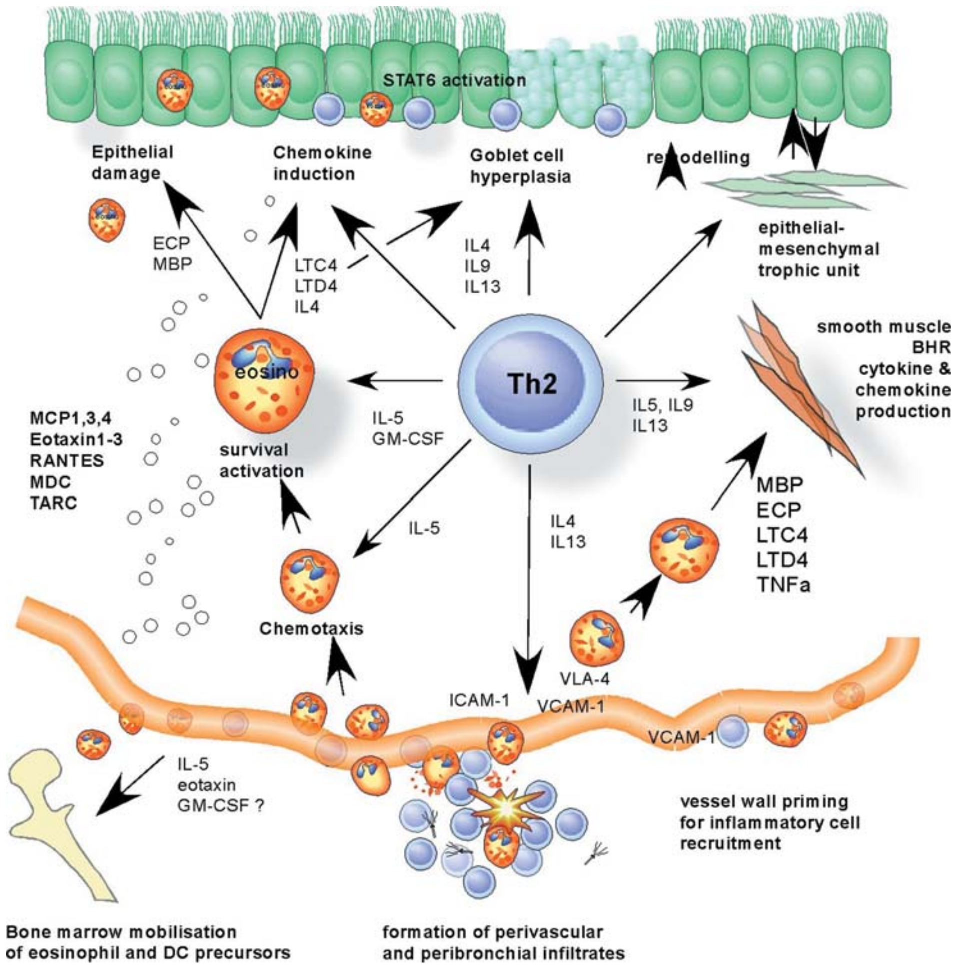

Fig. 50.1 Effector functions of Th2 cells. Effector Th2 cells produce several cytokines and chemokines which exert their effects on eosinophils, airway smooth muscle, epithelium and endothelium, resulting in several pathologic characteristics of asthma.

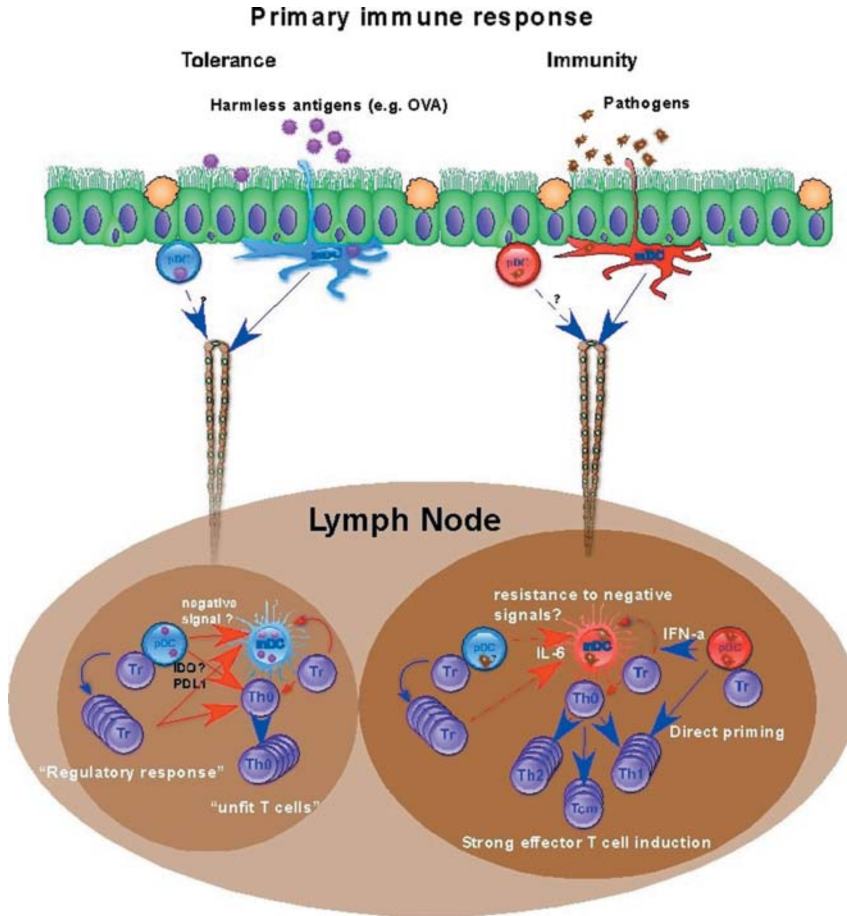

Fig. 50.2 Immune regulation by mDCs and pDCs in the lung. Under steady-state conditions (left), in the absence of accompanying danger signals in the lung, inhaled antigens are picked up by mDCs and pDCs, which take the antigen to the mediastinal nodes. Here, partially mature mDCs induce a short lived boost of division in antigen specific T cells, but these T cells fail to differentiate into effector cells and die. Some T cells might also differentiate into Treg cells. Plasmacytoid DCs in the draining node influence the generation of T effector cells from dividing T cells probably by giving negative signals (IDO, PDL-1) to T cells and mDCs. At the same time they alo generate Treg cells. Under inflammatory conditions (right), mDCs now arrive in the draining nodes as fully mature cells, resitant to Treg suppression. Ag specific T cells again undergo proliferation, but this time generating effector cells. On the other hand, pDCs also acquire a mature phenotype and prime Ag specific T cells to become effector cells as well.

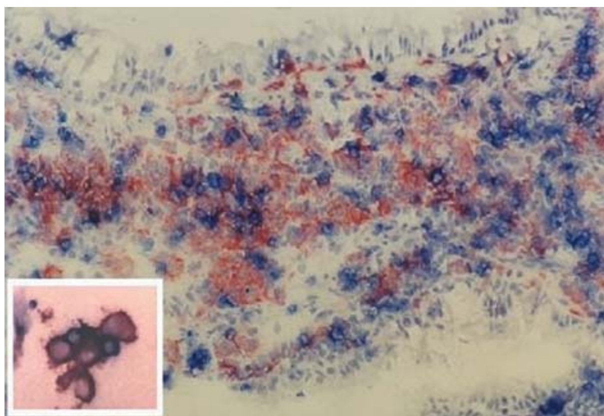

Fig. 50.3 Staining of $CD4^{+}$ T cells (membrane bound blue) and $CD11c^{+}$ DCs (red) in the lung of OVA sensitized and challenged mice visualizes co-localization of $CD4^{+}$ T cells and $CD11c^{+}$ DCs within peribronchial sites of inflammation. Goblet cell hyperplasia is seen.

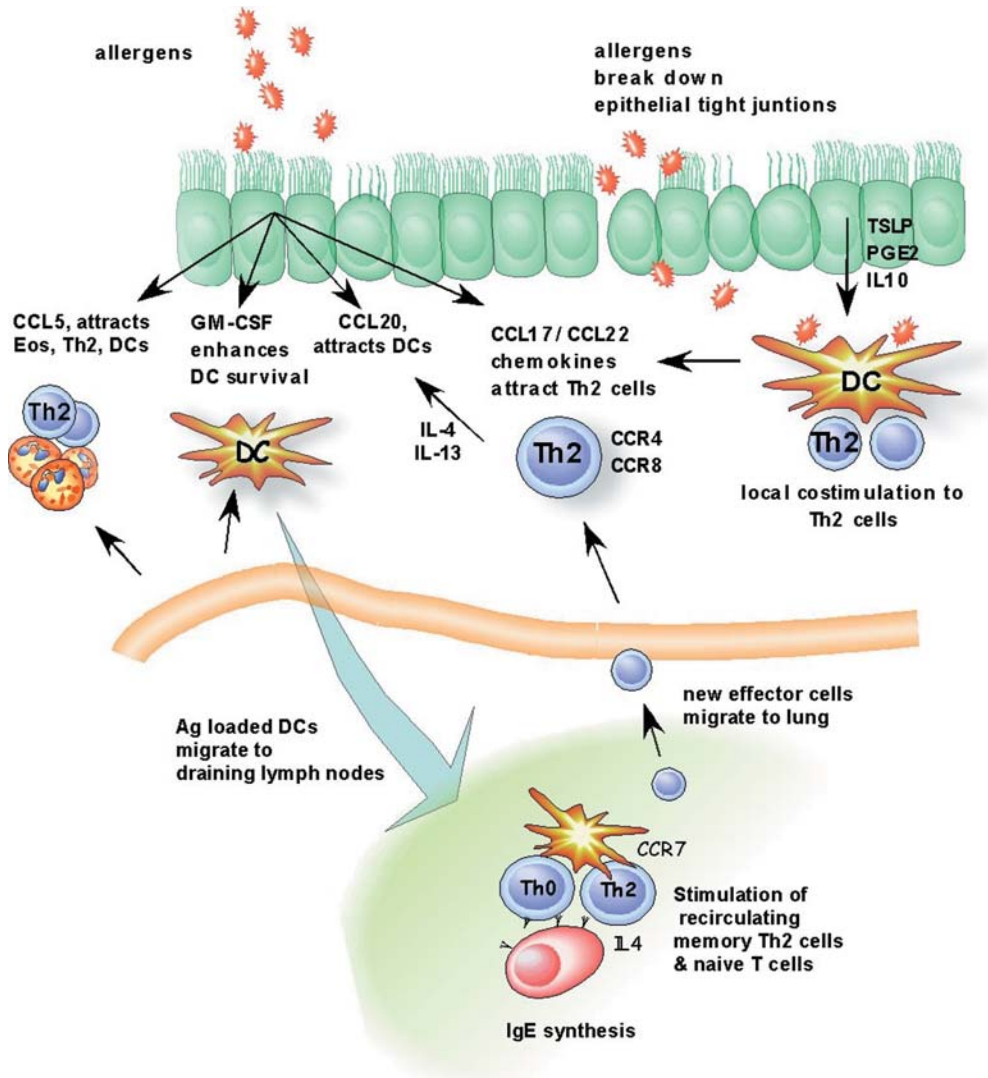

Fig. 50.4 Interaction between epithelial cells and dendritic cells during established inflammation. Allergens stimulate epithelial cells to release chemokines and growth factors for DCs, Th2 cells and eosinophils. The recruited DCs are also stimulated by allergen and produce even more chemokines for Th2 cells. Locally attracted Th2 cells interact with DCs in the airways, leading to local DC maturation and T-cell co-stimulation of effector cytokine production. These activated Th2 cells eventually control the inflammatory process by activating eosinophils and mast cells and by feeding back on the epithelium and DCs.cell.

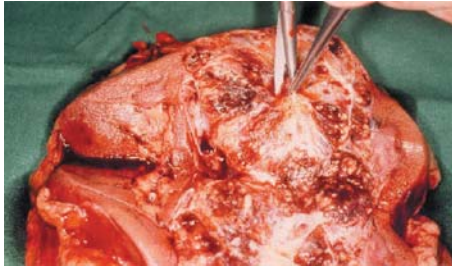

Fig. 53.1 Renal cell carcinoma: primary tumor in the kidney.

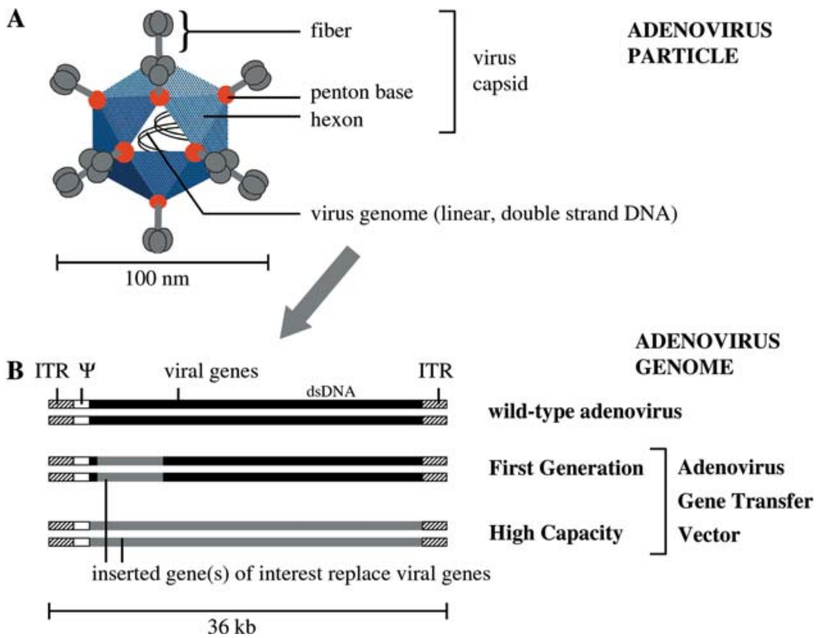

Fig. 55.2 Capsid (A) and genome (B) structure of adenoviruses and adenovirus vectors. The adenovirus capsid is an icosahedron that consists of 20 triangular surfaces and 12 vertices. It is built of 240 hexon proteins, 12 penton bases, 12 fibers and minor capsid proteins. The virus genome is a double strand DNA of approx. 36 000 base pairs with inverted terminal repeats (ITRs), a packaging signal (ψ) and the virus genes. The ITRs are required for replication of the virus genome, and ψ mediates incorporation of replicated genomes into pre-formed capsids. In first generation adenoviral gene transfer vectors the essential viral E1 genes (and E3 genes) are replaced by the transgene of interest. Hence, these vectors are replication-deficient and are produced in E1-transcomplementing cell lines. All viral genes are deleted in high capacity adenovirus vectors, but they retain ITRs and ψ. High capacity vectors are produced by co-infection with helper adenoviruses, which complement the viral gene products and need to be separated from the high capacity vectors after production.

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