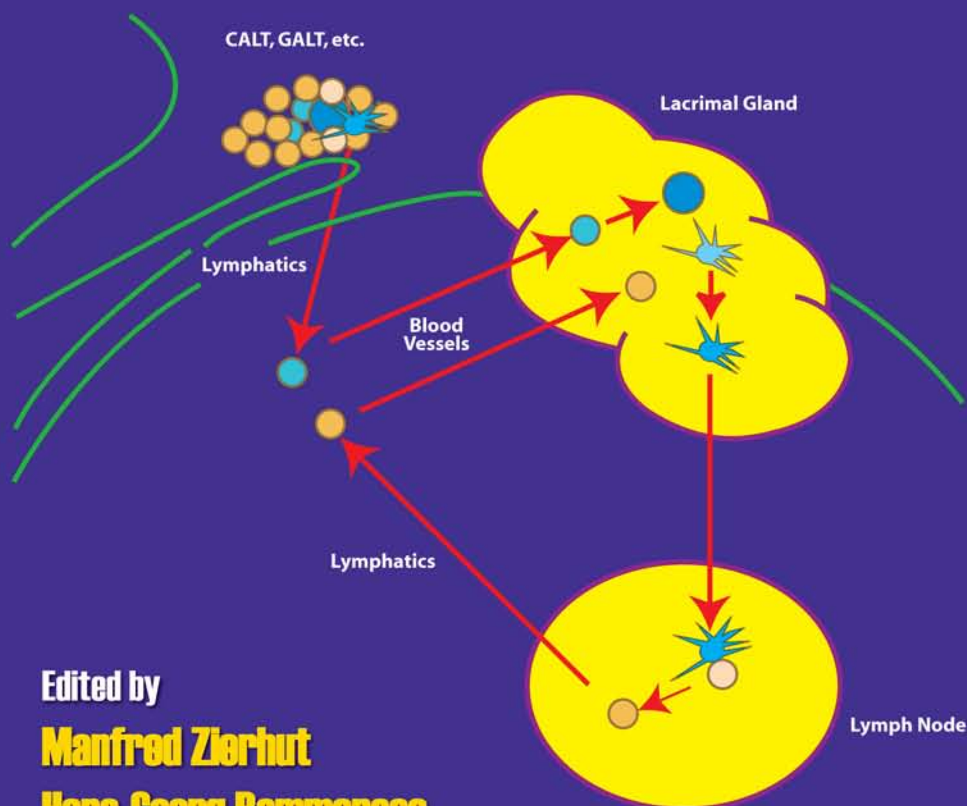
Antigen-Presenting Cells and the Eye



Edited by

Manfred Zierhut

Hans-Georg Rammensee

J. Wayne Streilein

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Edited by

Manfred Zierhut
University Eye Hospital, Tübingen, Germany

Hans-Georg Rammensee
University of Tübingen, Germany

J. Wayne Streilein
Schepens Eye Research Institute
Harvard Medical School
Boston, Massachusetts, USA

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Dedication

During the course of one's life, each of us has been influenced by a special person who has had an enormous impact in shaping who we are. For some, it was a teacher who stimulated our interests at a crucial time in our education. For others, it was a coach who gave us confidence and challenged us to reach higher. For hundreds of us, it was Wayne Streilein, who was a teacher, mentor, collaborator, colleague, and, most of all, a devoted friend.

Wayne possessed too many attributes to summarize in this brief overview, but let me highlight a few special qualities that have had a lasting impact on me, and I am sure hundreds of others. Wayne had the capacity to evoke interesting insights from everyone, whether it was a Nobel Laureate, such as Sir Peter Medawar, or the animal technician who changed the bedding in the mouse cages. Wayne would engage each of us in thought-provoking conversations and we would walk away feeling as if we were the most important person in his life and that we possessed profound insights into issues that we had not previously contemplated. When he entered a room, the conversations would soon elevate to a higher level, and each person would find himself feeling that he had something important to add to the discussions. He simply brought out the best in everyone.

Wayne had the same impact on the Ettal Research Workshops. He not only offered brilliant perspectives on the topic under discussion, but, equally important, he evoked insights from the participants that they had not previously considered. He created a synergism that energized each workshop and contributed to its success. His untimely death has created a void in the Ettal Workshops, and each of us who had the privilege to know him, still miss him immensely. It is with profound affection and admiration that we dedicate the proceedings of this workshop to his memory.

Jerry Niederkorn

Manfred Zierhut
Hans-Georg Rammensee

Preface

Antigen-presenting cells are indispensable for mediating the induction of a specific immune reaction. Various populations that differ in their location and activation grade have already been described.

This book provides a general evaluation of our understanding of antigen-presenting cells, and assesses their importance for the physiological and pathological condition of the eye. Autoimmune disorders that often lead to severe impairment of the eye's functions, for example, can be invoked by the presentation of self peptides by the antigen-presenting cells to the T-cell receptor complex. An analysis of this cascade may help to identify the initiating autoantigens. Depending on the activation status, dendritic cells can induce a T-cell reaction or, in contrast, even induce tolerance.

Until recently, antigen-presenting cells were thought to play a limited role only in the external segment of the eye, but the use of more refined detection methods has revealed a whole spectrum of different dendritic cells that are localized in the iris and the choroid. As far as the lacrimal gland and the anterior segment are concerned, the research is concentrating on the characterization of factors influencing ocular antigen-presenting cells. In addition, the role of antigen-presenting cells in the mucosa-associated lymphoid tissue in the physiological and pathological state, as found in the dry eye syndrome and infectious disorders, is under investigation. The significance of antigen-presenting cells in corneal disease, especially in the case of transplantation, is also of major importance. In recent years, multiple new subgroups of antigen-presenting cells have been detected in the cornea, but at the present time their respective role still remains unclear.

Antigen-presenting cells of the posterior eye segment are becoming the focus of increased interest because they appear to be strongly involved in two major disorders of the eye: uveitis and age-related maculopathy.

We hope that by summarizing our knowledge and by stimulating research in the field of antigen-presenting cells in the eye, this book contributes to a better understanding of the protective role of antigen-presenting cells and to the development of new therapeutics that incorporate these fascinating cells.

Manfred Zierhut
Hans-Georg Rammensee

Contents

Preface .... *v*
Contributors .... *xi*

1. **Dendritic Cell and Natural Type I Interferon-Producing Cell Development** *1*
Markus G. Manz

2. **Antigen Presentation by Human Leukocyte Antigen Molecules—One of the Keys for Understanding the Etiology of Autoimmune Disease?** *9*
Hans-Georg Rammensee

3. **Antigen Presenting Cell Interactions with Cells During Anterior Chamber Associated Immune Deviation** *17*
Joan Stein-Streilein

4. **The Role of Dendritic Cell Migration for the Induction of Immunity and the Maintenance of Tolerance** *27*
Manfred B. Lutz

5. **The Activation Status of Dendritic Cells Is Crucial for Decision Making on Tolerance Versus Immunity** *37*
Karsten Mahnke and Alexander H. Enk

6. **Distribution of Antigen-Presenting Cells in the Eye** *45*
Paul G. McMenamin, Season Yeung, and Serge Camelo

7. **Phenotype and Distribution of Antigen-Presenting Cells in the Mouse and Human Eye** *71*
Bita Manzouri, Santa Jeremy Ono, and Masaharu Ohbayashi

8. **Eye-Associated Lymphoid Tissue in Dry Eye Syndrome** *83*
Fiedrich Paulsen, Kristin Jäger, Saadettin Sel, and Philipp Steven

9. **Lacrimal Epithelium Mediates Hormonal Influences on Antigen-Presenting Cells and Lymphocyte Cycles in the Ocular Surface System** *93*
Austin K. Mircheff, Yanru Wang, Magdalena Baladud de Saint Jean, Chuanqing Ding, and Joel E. Schechter

10. **Antigen-Presenting Cells and Molecular Mechanisms Underlying Induction of Immune Deviation** *121*
Sharmila Masli, J. Wayne Streilein, and A. Paiman Ghafoori

11. **Regulatory Dendritic Cells and Their Potential for Tolerance Induction** *131*
Audrey H. Lau and Angus W. Thomson

12. **Corneal Antigen-Presenting Cells: What Have We Learned from Transplantation?** *151*
Reza Dana

13. **Therapeutic Manipulation of Ocular Antigen-Presenting Cells in Corneal Transplantation** *157*
Jerry Y. Niederkorn

14. **The Role of Corneal Antigen-Presenting Cells in Herpes Simplex Keratitis** *165*
Robert L. Hendricks

15. **Antigen-Presenting Cells and the Eye: Bacterial and Parasitic Infections** *169*
Linda D. Hazlett

16. **Skin Allergy Versus Ocular Allergy** *183*
Natalija Novak and Thomas Bieber

17. **Antigen Presentation in the Eye: Uveitis** *189*
Janet Liversidge, Patrick Tighe, Andrew Dick, and John V. Forrester

18. Role for Ocular Antigen-Presenting Cells in Pigmentary Forms of Glaucoma *199*
Michael G. Anderson, J. Wayne Streilein, and Simon W. M. John

19. Association of Major Histocompatibility Class II Antigens with Core Subdomains Present Within Human Ocular Drusen *209*
Gregory S. Hageman and Robert F. Mullins

20. Role of Macrophages in Uveal Melanoma *217*
Martine J. Jager, Teemu Mäkitie, Päivi Toivonen, and Tero Kivelä

Index *227*

Contributors

Michael G. Anderson Department of Molecular Physiology and Biology, University of Iowa, Iowa City, Iowa, U.S.A.

Thomas Bieber Department of Dermatology, University of Bonn, Bonn, Germany

Serge Camelo School of Anatomy and Human Biology, University of Western Australia, Crawley, Western Australia, Australia

Reza Dana Laboratory of Corneal Immunology, Schepens Eye Research Institute, Cornea Service, Massachusetts Eye and Ear Infirmary, and Department of Ophthalmology, Harvard University, Boston, Massachusetts, U.S.A.

Magdalena Baladud de Saint Jean Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

Andrew Dick Bristol Eye Hospital, Bristol, U.K.

Chuanqing Ding Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

Alexander H. Enk Department of Dermatology, University of Heidelberg, Heidelberg, Germany

John V. Forrester Institute of Medical Sciences, University of Aberdeen, Foresterhill, U.K.

A. Paiman Ghafoori Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

Gregory S. Hageman Department of Ophthalmology and Visual Science, University of Iowa, Iowa City, Iowa, U.S.A.

Linda D. Hazlett Department of Anatomy and Cell Biology, Wayne State University, Detroit, Michigan, U.S.A.

Robert L. Hendricks Department of Ophthalmology, Immunology, and Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

Kristin Jäger Department of Anatomy and Cell Biology, Martin-Luther-University of Halle-Wittenberg, Halle, Germany

Martine J. Jager Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands

Simon W. M. John The Jackson Laboratory, Howard Hughes Medical Institute, Bar Harbor, Maine, U.S.A.

Tero Kivelä Department of Ophthalmology, Helsinki University Central Hospital, Helsinki, Finland

Audrey H. Lau Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

Janet Liversidge Institute of Medical Sciences, University of Aberdeen, Foresterhill, U.K.

Manfred B. Lutz Institute for Virology and Immunobiology, University of Wuerzburg, Wuerzburg, Germany

Karsten Mahnke Department of Dermatology, University of Heidelberg, Heidelberg, Germany

Teemu Mäkitie Department of Ophthalmology, Helsinki University Central Hospital, Helsinki, Finland

Markus G. Manz Institute for Research in Biomedicine (IRB), Bellinzona, Switzerland

Bita Manzouri Department of Ocular Immunology, Institute of Ophthalmology, University College London, London, U.K.

Sharmila Masli Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

Paul G. McMenamin School of Anatomy and Human Biology, University of Western Australia, Crawley, Western Australia, Australia

Austin K. Mircheff Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

Robert F. Mullins Department of Ophthalmology and Visual Science, University of Iowa, Iowa City, Iowa, U.S.A.

Jerry Y. Niederkorn Departments of Ophthalmology and Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas, U.S.A.

Natalija Novak Department of Dermatology, University of Bonn, Bonn, Germany

Masaharu Ohbayashi Emory Eye Center, School of Medicine, Emory University, Atlanta, Georgia, U.S.A.

Santa Jeremy Ono Emory Eye Center, School of Medicine, Emory University, Atlanta, Georgia, U.S.A.

Fiedrich Paulsen Department of Anatomy and Cell Biology, Martin-Luther-University of Halle-Wittenberg, Halle, Germany

Hans-Georg Rammensee Departments of Cell Biology and Immunology, University of Tübingen, Tübingen, Germany

Joel E. Schechter Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

Saadettin Sel Department of Ophthalmology, Martin-Luther-University of Halle-Wittenberg, Halle, Germany

Joan Stein-Streilein Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

Philipp Steven Department of Ophthalmology, UK-SH, Campus Lübeck, Lübeck, Germany

J. Wayne Streilein† Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

Angus W. Thomson Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

Patrick Tighe Department of Immunology, Queens Medical Center, Nottingham, U.K.

Päivi Toivonen Department of Ophthalmology, Helsinki University Central Hospital, Helsinki, Finland

Yanru Wang Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

Season Yeung School of Anatomy and Human Biology, University of Western Australia, Crawley, Western Australia, Australia

†Deceased.

1

Dendritic Cell and Natural Type I Interferon-Producing Cell Development

Markus G. Manz
Institute for Research in Biomedicine (IRB), Bellinzona, Switzerland

INTRODUCTION

The differentiation of hematopoietic stem cells (HSCs) to mature cells is a lineal process, characterized by a stepwise loss of self-renewal capacity, and by final restriction to one mature cell type. HSCs, as well as developmental intermediates with limited cellular expansion potential and restriction to specific mature cell lineages, were isolated to high purity (for review, see 1,2). Consecutively, a phenotypic and functional defined hematopoetic developmental tree has been suggested: Long-term HSCs (LT-HSCs) give rise to short-term HSCs (ST-HSCs), which generate non–self-renewing, multi-potent progenitors (MPPs), which further develop to either clonal common lymphoid progenitors (CLPs) and subsequently to pro T cells or pro B cells, or to clonal common myeloid progenitors (CMPs) and subsequently to granulocyte/macrophage progenitors (GMPs) or megakaryocyte/erythrocyte progenitors (MEPs), all of which have been defined in both mice and humans (3–9). Those progenitors each have potent proliferative potential, generating plenty of progeny; however, they do not self-renew, and offspring cells are only generated in a single burst.

DENDRITIC CELL AND TYPE I INTERFERON-PRODUCING CELL DEVELOPMENT

Dendritic cells (DCs) as well as natural type I interferon-producing cells (IPCs, also called plasmacytoid dendritic cells, PDCs) are relatively recently described

cells of the hematopoietic system (1). With the exception of Langerhans cells (LCs) (10) and possibly some other non-lymphatic tissue DCs, most DCs and IPCs have a short in vivo turnover time of maximally two weeks (11,12). Thus, these cells need to be regenerated continuously from HSCs, a process that must be tightly regulated. Multiple DC types, differing in phenotype, localization, and function, were identified over the last few decades (13,14). With the recent phenotypic and functional identification of IPCs in both humans (15–17) and mice (18–20), and the finding that these cells are capable of differentiating to DCs, the heterogeneous group of DCs was further enlarged (21). Over the last few years we and others were able to elucidate some of the developmental pathways of these rare cells. Most of these findings are summarized in recent reviews (e.g., 14,21–24). Thus, I focus on our findings on DC and IPC development from mouse bone marrow or human cord-blood early-progenitor cells.

Regarding DC development, two opposing models were suggested: a "specialized lineage" model, where different DC subtypes are determined at the level of early hematopoietic progenitors and thus belong to different hematopoietic lineages; and a "environmental instruction" model, where different DC subtypes belong to the same hematopoietic lineage and, upon local influences, are determined at the level of an immediate DC precursor. The notion of specialized DC lineages was supported by several studies in mice and men: first, it was shown that mouse thymocyte progenitors are capable of producing CD8a-expressing DCs in vivo, a DC population that constitutes most DCs in mouse thymus and about 30% of DCs in secondary lymphoid organs. Thus, a T-cell-associated CD8α "lymphoid" DC lineage was suggested (25,26), a concept that seemed to be further enhanced by the findings that these cells do not need granulocyte–macrophage colony-stimulating factor (GM-CSF) for DC differentiation in vitro (27), and that several transcription factor deficient mice lack only CD8α negative DCs (28–30). Second, human (and later also mouse) IPCs were suggested to be of lymphoid lineage origin because human IPCs express CD2, high CD4, CD5, and CD7, but not CD11c, CD13, and CD33 (15,21), because they express T- and B-cell development-associated mRNA transcripts (31–34), and because GM-CSF does not promote their development (35,36). However, although these observations added significantly to the understanding of critical cytokines and transcription factors for DC and IPC development, the conclusions on lineage associations remained indirect.

To more directly test DC and IPC lineage associations, developmental capacities from each above-mentioned lineage restricted progenitor populations were tested in vitro and in vivo. We and others came to several unexpected findings: (*i*) mouse CLPs, as well as CMPs, generate CD8α positive and CD8α negative DCs in vivo (37–39); (*ii*) DC differentiation activity is preserved in early T-cell progenitors, declining along T-cell maturation, as well as in GMPs (38,39); (*iii*) irrespective of the progenitor transplanted, we preferentially found development of CD8α positive DCs in lethally irradiated animals (37,38); (*iv*) the

classical DC and IPC developmental capacity of progenitors is overlapping (40–42); (*v*) if DC and IPC reconstitution capacities of progenitors reflect in vivo, steady-state DC and IPC development, most secondary-tissue DCs and IPCs are of myeloid origin, while myeloid and lymphoid progenitors reconstitute about half of thymus DCs and IPCs each, respectively (37,38,40,42); (*vi*) definitive B-cell or MEP commitment terminates both DC and IPC developmental capacities (38,40); (*vii*) finally, human myeloid and lymphoid progenitors show similar DC and IPC developmental capacities as mouse progenitors (43). Taken together, these experiments uncovered an unexpected redundancy in DC and IPC development from both lymphoid and myeloid progenitor cell populations.

We thus were interested in determining what might define the capacity of these progenitors to receive and execute signals that drive DC and IPC development. We and others looked at the flt3-receptor/flt3-ligand, a nonredundant cytokine receptor/ligand pair in DC and IPC development. Flt3-ligand knockout mice and mice with hematopoietic system restricted Stat3 deletions show massively reduced DCs and IPCs (44,45); flt3-ligand injection or over-expression of flt3-ligand increases DCs and IPCs in mice and men (19,46–53); moreover, flt3-ligand is capable of driving in vitro differentiation of both human and mouse DCs and IPCs (35,43,54). The receptor for flt3-ligand, flt3, was shown to be expressed on ST-reconstituting HSCs in mice (55,56). We further evaluated expression of flt3 along the hematopoietic tree. Flt3 is transiently upregulated from ST-HSCs on most common lymphoid and myeloid progenitors, but is downregulated in definitive B-cell, T-cell, and MEP lineage commitment (49); while flt3 is not expressed on other steady-state hematopoietic cell lineages, it is expressed by lymphoid tissue DCs and IPCs (49). Furthermore, both lymphoid and myeloid offspring DCs are increased in flt3-ligand injected animals (49). Thus, DC and IPC development is confined to flt3 expressing hematopoietic progenitor cells, and flt3-ligand drives DC and IPC development along both pathways (41,49).

CONCLUSION

We thus further tested whether flt3 would be capable of delivering an instructive signal for DC and IPC development. Indeed, over-expression of flt3 in flt3 negative progenitors rescues DC and IPC development to levels of flt3 positive progenitors. This process involves upregulation of DC- and GM-development affiliated genes (57). These findings suggest that flt3 signal strength might regulate DC and IPC development. We therefore propose a steady-state “flt3-license pathway” for DC and IPC development, where differentiation of these cells from flt3 positive progenitors is possible as long as no competing signal shuts it down. It is important to stress that this model might only reflect the situation in steady state. During inflammatory stress, GM-CSF, IL-4, and TNF-α likely become important cytokines that guide monocytes and macrophages to differentiate to DCs. Indeed, in vivo DC development from mouse monocytes was not observed in steady state, but only upon inflammation (58–60). Taken together, the

flt3-pathway would be dominant in steady state, while the GM-CSF-pathway might be a major pathway upon inflammation.

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2

Antigen Presentation by Human Leukocyte Antigen Molecules—One of the Keys for Understanding the Etiology of Autoimmune Disease?

Hans-Georg Rammensee

Departments of Cell Biology and Immunology, University of Tübingen, Tübingen, Germany

INTRODUCTION

Most autoimmune diseases are human leukocyte antigen (HLA)-associated. The reason for this is most likely the individualization of the immune response by HLA-mediated antigen presentation to T cells. The following provides a basic introduction to this phenomenon.

With few exceptions, all nucleated human cells express HLA class I molecules on their surfaces. These consist of a heavy chain encoded by one of the three HLA class I loci, HLA-A, HLA-B, or HLA-C on chromosome 6, and a noncovalently-associated light chain, beta-2-microglobulin, encoded on chromosome 15. HLA class II molecules, in contrast, are only expressed on a limited set of cells, most notably on B cells, macrophages, dendritic cells (DC), and thymic epithelium. Some cells, such as epithelial cells, can be induced to express HLA class II in response to inflammatory conditions, i.e., exposure to IFNγ, whereas most cell types will never be HLA class II-positive. HLA class II molecules are dimers of an alpha-chain and a beta-chain, encoded on the HLA-DR, -DQ, and -DP loci on chromosome 6. The intrinsic biological difference between HLA class I and class II is to be found in the responsibility for reporting information to different subsets of T cells.

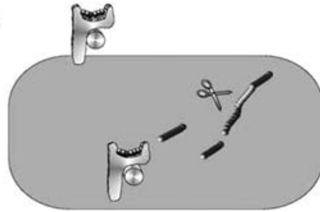

Figure 1 A protein molecule inside a cell is fragmented by enzymes; some of the fragments associate with an HLA molecule, and the resulting noncovalent combination of fragment and HLA class is brought to the cell surface, where it might be recognized by a T cell. *Abbreviation*: HLA, human leukocyte antigen.

HLA class I delivers information to CD8 T cells; HLA class II delivers to CD4 T cells. The common feature of both types of molecules is shown in Figure 1.

HLA CLASS I

An artist's impression of virus-antigen presentation by HLA class I molecules to a cytotoxic CD8 T cell can be seen in Figure 2. The cell lying below is being infected by a virus. Some of the viral proteins (coded in red) are fragmented by the barrel-shaped enzyme complex, the proteasome, located in the cytosol. The fragments (peptides) are then transported into the lumen of the endoplasmic reticulum (ER) by a specialized molecule, the transporter associated with antigen processing (TAP). Inside the ER, newly synthesized HLA class I molecules wait to be loaded with peptides. Once fully loaded, these then travel to the cell surface. The cell looking down from above is a cytotoxic CD8 T cell that recognizes the red (virus-derived) peptide plus HLA class I by means of its T-cell receptor. As a consequence, the T cell releases its cytotoxic molecules, which include pore-forming perforins. This leads to perforation and, ultimately, to the death of the attacked cell. The blue protein and the blue peptides symbolize the normal self-protein compartment and peptides derived thereof. Each of the approximately 10,000 different normal proteins inside any cell is subject to continuous recycling, and samples of the resulting peptides are loaded onto HLA class I molecules (1). Thus, a normal cell presents up to 10,000 different peptides, which essentially represent the entire protein content on its surface, a notion central to considerations on autoimmunity. Usually, however, T cells do not attack such self-peptides by virtue of negative selection of self-reactive T cells during their development in the thymus (2), and by the absolute requirement of a contribution of the innate immune system to the primary activation of a naïve T cell (Fig. 2) (3).

HLA CLASS II

An overview of antigen presentation by HLA class II (4–6) is displayed in Figure 3. Newly synthesized α and β chains are chaperoned in the ER by a third chain, the

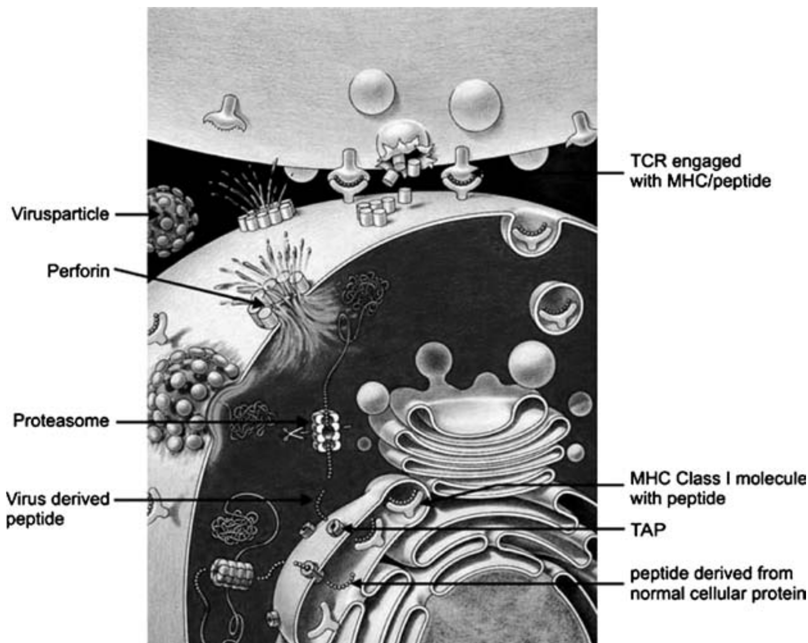

Figure 2 (*See color insert.*) Cellular proteins, including viral proteins (coded in red), are degraded by the proteasome. The resulting peptides are transported to the endoplasmatic reticulum (ER) lumen via the transporter associated with antigen processing (TAP), and loaded onto MHC class I molecules (HLA class I for human cells). Loaded major histocompatibility complex (MHC) I molecules are then transported to the cell surface to be screened by T cell receptors. *Source*: Drawn by Klaus Lamberty, Tübingen.

invariant chain, Ii. A second function of Ii is to clog the peptide-binding site so that peptides, present in the ER, cannot associate with HLA class II. The third function of Ii is to provide an addressing signal that directs HLA class II molecules into the peptide-loading compartment, an endosome-like structure. There, further chaperones, including HLA-DM, as well as proteolytic enzymes such as cathepsins, remove and fragment Ii and allow peptides produced by cleavage of endosomal proteins to bind to the peptide-binding site. Finally, the peptide-loaded HLA class II molecule is translocated to the cell surface to be recognized by T cells. Note that peptides presented by HLA class II can be derived from phagocytosed proteins as well as from normal cellular proteins present in endosomes, including all membrane proteins.

CROSS-TALK

The antigen presentation pathways for HLA class I and HLA class II loading use entirely different compartments and enzymes, yet there is ample cross-talk. Two mechanisms appear to be of special significance in this process:

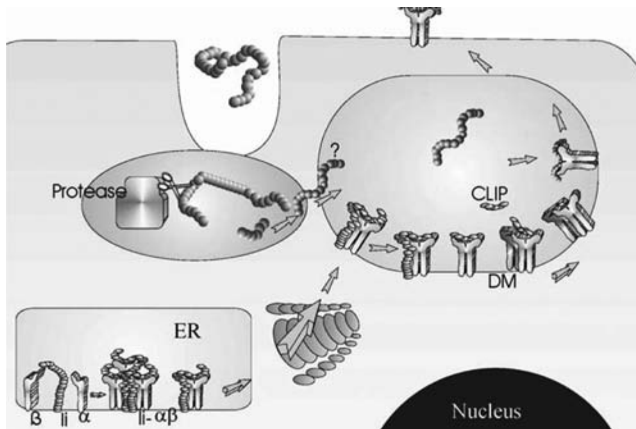

Figure 3 The two chains for major histocompatibility complex (MHC) class II (HLA class II for human cells), α and β, are synthesized into the endoplasmic reticulum (ER), and complexed by the invariant chain (Ii). These complexes are then translocated to the MHC class II loading compartment, where Ii is enzymatically degraded. The remaining peptide of Ii, class II associated invariant chain peptide (CLIP), is then removed by DM, a chaperone, and finally replaced by the nominal antigen peptide that can be derived from a phagocytosed particle. Fully assembled MHC class II molecules are translocated to the cell surface to be screened by T cell receptors.

1) Presentation of peptides derived from phagocytosed proteins by HLA class I (instead of HLA class II, as would be the default way), which is a process also known as "cross priming." This is observed in DCs; in mice, there is a subset of DCs, the CD8+ DCs, which are capable of cross-presentation (6). Human DCs are also capable of cross-presentation, but a special subset that possesses this ability is not known. It is thought that cross-presentation, for example, of viral antigens on HLA class I molecules of DCs is important for the first priming of naive T cells.
2) Presentation of peptides derived from cytosolic or nuclear proteins on HLA class II molecules by autophagy (7,8). This is an emergency process used by starving cells to maintain a supply of amino acids: small volumes of cytosol are engulfed by membranes and fused to lysosomes; this is followed by digestion of its contents. It is thought that a baseline level of this activity is maintained by cells at all times, so that each HLA class II-expressing cell will present some normal cytosol and nucleus-derived peptides—again a notion important for considerations of autoimmunity.

HLA SPECIFICITY

As implied above, HLA molecules are able to bind peptides, and as such are peptide receptors. HLA peptide specificity is quite unique in that each particular HLA

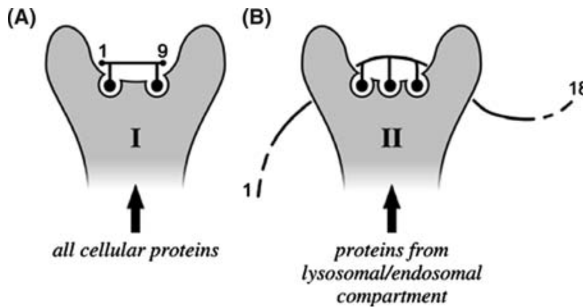

Figure 4 Binding protein chain in (**A**) HLA class I and (**B**) HLA class II molecule. While HLA class I molecules can process all cellular proteins, HLA class II molecules only process protein from the lysosomal or the endosomal compartment.

molecule, class I or class II, can bind thousands of different peptides as long as they have certain structural features in common (9). Typically, HLA class I molecules bind peptides of 9 amino acids (8–11), whereby two or three of the positions are occupied by particular amino acids (10). As we know well from crystal structures (11), the side chains of such residues are anchored in corresponding pockets of the HLA molecule (Fig. 4A). Each HLA molecule (allelic product) has its own particular specificity that is defined by position (e.g., at position 2 and at the C-terminus) and identity (e.g., leucine or a similar aliphatic residue). One example for peptide specificity of this kind is given for two HLA class I molecules (Fig. 5).

HLA class II molecules present peptides with similar characteristics (Fig. 4B), whereby the length of presented peptides ranges from 12 through 20 amino acids. A selection of well-known HLA class I and class II ligands and T-cell epitopes can be found in Figure 6 (12,13) [a database for such ligands is at (14)].

HLA POLYMORPHISM

HLA class I presents the most polymorphic gene system in humans. For HLA-A, 341 different allelic products are known, 648 for HLA-B, and 419 for HLA-DRB (15). Each individual expresses his or her own individual combination of HLA genes, consisting approximately of 6 HLA class I and >6 HLA class II genes. Since each of the allelic products has its own individual peptide specificity, it follows

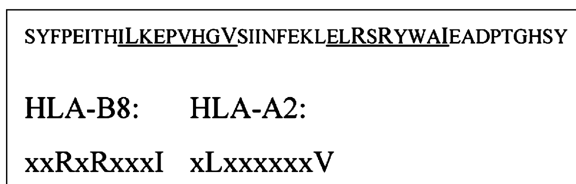

Figure 5 Peptide selection by HLA class I-molecules. Further explanation in the text.

HLA-A*0201	GILGFVFTL	Influenza A MP 58 - 60
	ILKEPVHGV	HTV RT 476 - 484
	NLVPMVATV	HCMV PP65 495- 504
HLA-DRB1*0701	P G P L R E S I V C Y F M V F L Q T H I EBV EBNA-1 551-570	

Figure 6 Selection of HLA-class I and class II ligands and T cell epitopes. *Source*: From Ref. 12.

that each HLA molecule presents a different set of peptides, even, for example, from the very same viral protein. Due to this fact, antigen presentation and, as a consequence, the immune response of each human being are individualized. This is illustrated by the following example using Influenza A-nucleoprotein. T cells from persons with the indicated HLA alleles recognize the following peptides:

- HLA-A1 peptide CTELKLSDY (44–52)
- HLA-A3 peptide ILRGSVAHK (265–274)
- HLA-A68 peptide KTGGPIYKR (91–99)

Thus, HLA polymorphism has functional consequences on T-cell mediated immunity. While the impact of individualized antigen presentation and recognition on the triggering of autoimmune T cells is evident as well, it is far from being clear which precise events are responsible for the association between autoimmune disease and HLA polymorphism. If we assume that a self-peptide being recognized by an autoreactive T cell under activating conditions (see below) initiates autoimmune disease, then several possible reasons for this have to be considered:

1) Insufficient negative thymic selection of the self-reactive T cells as a consequence of weak peptide/HLA binding
2) Activation of the self-reactive T cell in the periphery by a strongly-binding self-peptide; e.g., after enhanced presentation of the self-peptide in question due to environmental influences
3) Primary activation of a self-reactive T cell upon costimulatory conditions during an infection (see below)

A similar consideration can be made for the possibility that autoimmunity is normally inhibited by regulatory T cells (Treg) that are believed to be antigen-specific, at least during their induction phase (16). Two of the possibilities cited above for autoimmune T cells would then apply but with the opposite meaning: (*i*) complete negative selection of the precursor of a self-specific Treg on account of high affinity peptide/HLA binding, or (*ii*) insufficient activation of the self-specific Treg in the periphery by a weakly binding self-peptide.

THE INNATE IMMUNE SYSTEM CONTROLS T-CELL ACTIVATION

A naïve T cell (one that has never met its antigen before) can only be activated if it receives two signals (17). One signal is the engagement of its T-cell receptor with the fitting peptide/HLA combination. The other is the costimulation signal provided by engagement of the CD28 molecule on the T cell's surface with B7 or CD80 (3,17,18) on the antigen-presenting cell (APC), which is an activated dendritic cell in most cases.

Resting DCs, located in tissues, are unable to provide costimulation to T cells. Only if activated, for example, by a Toll-like receptor (TLR) binding to its ligand, can the DC start the process that enables it to mature into a competent APC (Fig. 7). The changes involved include a decrease in phagocytosis, upregulation of major histocompatibility complex (MHC) I and II molecules, expression of costimulatory molecules, and migration to a local lymph node. The eleven known TLRs include specificities for a number of structures unique to groups of infectious agents. These structures include lipopolysaccharide from gram-negative bacteria (TLR4), lipopeptides from other bacteria (TLR2), and double-stranded RNA from viruses (TLR3). Thus, the control of naïve T cell activation by the innate immune system normally avoids the activation of T cells escaping central tolerance induction.

CONCLUDING REMARKS

Activation of a T cell requires the existence of a peptide/MHC combination that fits to its TCR as a signal and the presence of this antigen on an activated, costimulatory APC. If it is true that recognition of self-peptides on HLA molecules is involved in

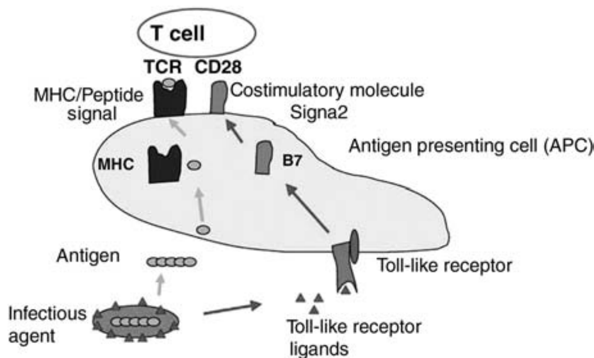

Figure 7 Induction of adaptive immune response. A resting antigen presenting cell (APC) does not express costimulatory molecules like B7. Upon contact with an infectious agent, substances thereof bind to their matching toll like receptors on the APC and induce cellular signalling, which initiates expression of B7. In parallel, antigen from the infectious agent is acquired by the APC and presented by the major histocompatibility complex (MHC) molecules on the cell surface. Thus, the activated APC provides both MHC-presented antigen and costimulation.

the etiology of autoimmunity, one has to assume that an autoreactive T cell that has slipped through central-negative selection happens to be activated on a mature DC, presenting either the very same self-peptide during an infection, or presentation of a cross-reactive peptide derived from the infectious agent, and both in the absence of sufficient regulatory T cells. In conclusion, we know of a number of potential checkpoints that are possibly, or even most likely, involved in the etiology of autoimmune diseases and dependent on the particular interaction of peptides, HLA molecules, and T-cell receptors. Detailed knowledge of these interactions should help us to understand and, as a result, counteract autoimmune diseases.

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3

Antigen Presenting Cell Interactions with Cells During Anterior Chamber Associated Immune Deviation

Joan Stein-Streilein
Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

THE DENDRITIC CELL AS AN ANTIGEN-PRESENTING CELL

In general, the subset of antigen-presenting cells (APCs) that is uniquely well equipped for presenting antigen to T cells and is regarded as sentinels for inducing immune responses is the dendritic cell (DC) subpopulation (1,2). T cells recognize antigens presented by major histocompatibility complex (MHC) molecules expressed on the APCs, and the subsequent interactions determine the differentiation pathway for the T cell. APCs are made up of a heterogeneous family of cells that is able to process both exogenous and endogenous antigens into 10–20 amino acid peptides and load them on to MHC molecules, which then traffic to the membrane for subsequent recognition by the antigen-specific T-cell receptor (3,4). APCs may be further classified into professional APCs (bone-marrow-derived DCs), which are able of activating and inducing clonal expansion of both naïve and memory T cells and nonprofessional APCs (B lymphocytes, monocytes, macrophages, endothelial cells), which are able to stimulate memory T cells but are poorly equipped to stimulate naïve cells to differentiate into effector cells. Within the tissues, the DC phenotype is immature but is capable of maturation if presented with “danger” signals (5–7). The immature phenotype also matures in

response to signals from innate cells [natural killer (NK), NKT, $\gamma\delta$ cells] (8). In return, the innate cell may become activated. Besides expression of co-receptors (CD80, CD86, Ox40 ligand, CD40), mature DCs exhibit decreased endocytosis of extracellular antigens, translocate the peptide-loaded MHC molecules into the plasma membrane, and display long-lasting peptide MHC complexes. Mature DCs also display increased membrane expression of chemokine receptor CCR7 (3), which responds to a gradient of chemokines released from the stromal cells residing in the T-cell areas of the secondary lymphoid organs (9).

Other APCs in the tissue include the tissue macrophages that, when activated, acquire a dendriform morphology. Whether macrophages become DCs is a matter of controversy. It is known that APCs in the tissue take up, process, and transport antigens to the secondary lymphoid organs. The state of the APC that sees the antigen is critical to the outcome of the immune response. When antigen is deliberately inoculated with adjuvants that contain "danger" signals, the APCs are apt to induce an inflammatory immune response.

The mature APCs travel to the spleen and are deposited in the marginal zone (MZ) of the spleen when they exit the blood through the central arteriole. A chemokine gradient encourages the inflammatory APCs and lymphocytes to move toward the T-cell areas of the lymphoid organ (10–12). The seductive chemokines that are produced by the stromal cells are CCL19 and CCL21, and the receptors expressed by the cells that will move into the T-cell areas are CCR7 and CXCR5 (13). The cells destined for the T-cell areas of the spleen leave the marginal zone area within four to six hours of their arrival.

The inflammatory APCs present antigen to T cells in the T-cell areas in the context of the Class II MHC. T cells are induced to proliferate and differentiate into T-effector or T-helper cells, following a series of other steps, including ligation of co-signaling surface molecules and binding of co-signaling cytokines for the efficient induction of the Th1 or Th2 type T-cell responses. Differentiated effector T cells modulate their chemokine receptors, and then leave the T-cell areas to percolate through the periphery in search of antigen.

CELLULAR INTERACTIONS DURING TOLERANCE INDUCTION

Central tolerance (14,15) differs from peripheral tolerance (16) in that the former occurs during fetal life and the latter during adult life. Moreover, there are multiple mechanisms for peripheral tolerance induction and the different models of peripheral tolerance may share some but not all the mechanisms of regulation for an unwanted immune response. During the adult life, active suppression of immune responses may be mediated by apoptosis, T-cell energy, or the development of T regulatory cells (Tregs) (16). The $CD4^+$ Tregs are reported both as being antigen specific and non-specific (17,18) but most reports of the $CD8^+$ Tregs are antigen specific. A large number of antigen non-specific $CD4^+CD25^+$ T cells are generated in the thymus, but the antigen specific $CD4^+CD25^+$ Tregs may also be induced in the periphery (19). Whether the non-specific and specific

$CD4^+CD25^+$ Tregs are part of the same lineage of regulatory cells has not been definitively determined.

While many studies are reported on the characteristics of the APC that presents antigen for T effector cell differentiation, there are few studies on the characteristics of APCs that present antigen for the development of T regulatory differentiation. In addition, the cellular interactions involved in peripheral tolerance induction are not as well detailed as during an immune inflammatory response. An exception is the cellular mechanism for the induction of tolerance through the eye that has been studied in a model of peripheral tolerance called anterior chamber associated immune deviation (ACAID). These studies suggest that a different process may exist for cellular interactions for tolerance induction and the interactions occur at a site different from where T effector cells develop. This chapter summarizes the literature and available information that address the cells and cellular interactions that are required for the generation of $CD8^+$ Tregs during the induction of peripheral tolerance via the eye and immune privileged site (20).

APC INTERACTIONS FOR THE GENERATION OF $CD8^+$ T REGULATORY CELLS

Two laboratory models of peripheral tolerance that are reported to generate efferent $CD8^+$ T regulatory cells are ACAID (21) and "Low dose oral tolerance" (LdOT) (22). An assumption held by many in the field of immunology is that peripheral tolerance occurs when the required co-signaling molecules for an immune response are missing. While this is certainly the case sometimes, it appears to not be the case for the induction of $CD8^+$ Tregs in ACAID. Strong evidence is now published that ACAID $CD8^+$ Tregs are produced by unique cross-talk between cells that aggregate within the MZ of the spleen (23,24). The tolerogenic signal that leaves the eye three days post antigen inoculation was first identified as an F4/80 $^+$ APC by Wilbanks et al. in the early 1990s (25,26). Wang et al. suggest that the eye-derived tolerogenic APCs traffic through the thymus on their way to the spleen (27). Faunce et al. showed that the presumed eye-derived $F4/80^+$ APCs aggregated in the MZ of the spleen after anterior chamber (AC). inoculation and peaked in numbers by seven days (23). Since many more $F4/80^+$ APC accumulated in the tolerogenic clusters than could have possibly come from the eye, these data support an earlier suggestion that the eye derived $F4/80^+$ APCs educated other $F4/80^+$ APCs to acquire the capability to induce Tregs rather than T-effector cells. Within the MZ, the F4/80 APCs interact with an invariant (*i*) NKT cell (24), MZ B cells (28), and $CD3^+$ T cells (presumably, both $CD4^+$ and $CD8^+$) (Fig. 1). It is also known that classical $CD4^+$ T cells are not needed for the generation of the ACAID $CD8^+$ Tregs since these Tregs have been generated in Class II knockout (KO) mice (29). While CD4 protein is expressed by the iNKT cell required for ACAID induction, the function of the CD4 protein on the NKT cell is unknown.

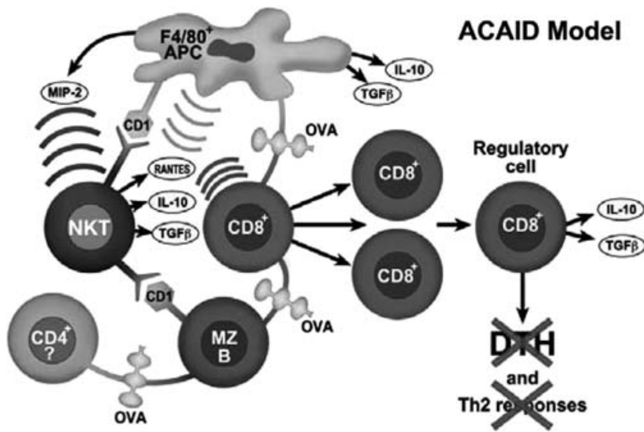

Figure 1 (*See color insert.*) Illustration of the cellular interactions during ACAID induction. The F4/80+ APC (light green) travels to the marginal zone of the spleen secreting MIP-2 chemokine along the way. The F4/80+ cell secretes IL-10 and TGF*ß*. The MIP-2 recruits NKT cells to the same region of the spleen. The TCR on the NKT cell binds to CD1d on the F4/80 cell and the MZ B cell (dark green). The interaction of the NKT cell with CD1d on the APC induces the production of RANTES that recruits more F4/80 APC and CD8+ T cells to the region. The NKT cell also produces immunosuppressive cytokines, IL-10 and TGF*ß*. Both the F4/80+ APC and the MZ B cell are capable of presenting the antigen (OVA) to the T cells in the cell cluster. In response to antigen presentation by the tolerogenic APC and the immunosuppressive environment, the CD8+ T cell differentiates into regulatory cells that suppress both Th1 and Th2 effector cell responses. This same process may be responsible for the development of ACAID CD4+ Tregs but has not been formally assessed. The suppressive milieu of the cell clusters in the MZ may mimic an immune privilege site in the spleen, ideally designed to induced T regulatory cells rather than effector cells. *Abbreviations*: ACAID, anterior chamber associated immune deviation; APC, antigen-presenting cell; MZ, marginal zone; NKT, natural killer T cells; MIP, macrophage inflammatory protein; TCR, T cell antigen receptor.

CHARACTERISTICS OF EYE-DERIVED TOLEROGENIC F4/80+ APCs

The F4/80+ APCs in the eye are exposed to a variety of immunosuppressive factors including TGFβ, VIP, CGRP, and αMSH (30). Because of the immunosuppressive environment, the "eye-derived" APCs expressed low amounts of CD40, little or no IL-12, and produce IL-10 and TGFβ (31). Since both IL-10 and TGFβ are autocrines that are able to induce their own production in cells that are exposed to them, the eye-derived APCs are capable of educating other cells to produce immunosuppressive factors (25,32–34).

In addition, tolerogenic "eye-derived" APCs also need to express the following surface molecules if they are to be efficient in inducing tolerance: CD1d, Class I, and F4/80. Furthermore, ACAID and LdOT cannot be induced in mice that are deficient in either CD1d or F4/80 unless the deficient animals are

reconstituted with cells that express the missing molecule (24,35). CD1d needs to be expressed so that the APC may interact with the invariant (i) TCR α chain of the iNKT cell (24). β2microglobulin deficient mice (lack Class I) are incapable of responding to AC inoculation of antigen with ACAID since both classical Class I and Class I-like CD1d are missing.

The ACAID inducing APCs are further distinguished by their expression of F4/80 protein. F4/80 protein is a molecule that has long been associated as a marker of tissue macrophages. The F4/80 molecule was identified over twenty years ago as a 160-kDa molecule expressed on the plasma membrane of restricted subsets of mouse macrophages, monocytes, and dendritic cells found throughout the lymphoid system and elsewhere in the body (36). The expression of its F4/80 molecule is known to fluctuate in response to BCG infection or IFN-γ (37). Additionally, the fact that it is known that F4/80 expression on activated Langerhans cells and other migrating APCs decreases, suggests a role for the molecule in cellular adhesion events (38). The F4/80 molecule was cloned and sequenced by McKnight and colleagues who showed that the molecule exhibited significant amino acid sequence homology to the seven transmembrane domains of both the EGF and hormone receptor superfamilies (39). Despite such molecular advances, the function of the F4/80 molecule has remained elusive. Because F4/80 protein expression is required, it is speculated that F4/80 might enhance adherence of the tolerogenic APC to the other cells (lymphocytes and stromal cells) in the marginal zone. On the other hand, signaling through F4/80 has not been ruled out (35).

Relevant to the requirement of these surface molecules in ACAID is the fact that both CD1d and F4/80 are needed for the generation of $CD8^+$ Treg cells in the peripheral tolerance model of LdOT as well as ACAID (35). Because of the correlation of CD1d and F4/80 in both ACAID and LdOT, it is presumed that similar interactions between the APCs, iNKT cells, T cells, and MZ B cells within the MZ of the spleen may occur in both models. This bold speculation, however, needs to be tested experimentally.

MOLECULES, CYTOKINES, AND CELLS NOT NEEDED FOR APC INTERACTIONS DURING TOLERANCE INDUCTION

In addition to learning about mechanisms of tolerance by the molecules that are needed for the induction of a peripheral tolerance response, it is worth knowing the molecules, cytokines, and cells that are not needed for the generation of the $CD8^+$ Tregs.

Since Class II deficient mice are unable to mount a delayed hypersensitivity (DH) response (they lack the classical $CD4^+$ T effector cells), these KO mice had never been tested for the development of ACAID. However, Nakamura used a local adoptive transfer assay to show that indeed, $CD8^+$ Tregs were generated following AC inoculation of antigen. Thus classical $CD4^+$ T cell help is not needed for the generation of ACAID $CD8^+$ Tregs (29).

Early in the study of ocular immunology, suppression of DH was almost synonymous with ACAID. During this time the idea arose that ACAID was a deviation

from a Th1 response to a Th2 response (40). But it now is known that after AC inoculation of antigen, not only are Th1 responses suppressed but Th2 inflammatory responses are regulated as well (41–43). To further test the role of Th2 responses in the generation of ACAID, Nakamura and colleagues showed that IL-14, IL-13, and Stat-6 were not needed for the generation of ACAID since all mice deficient in any one of these molecules were perfectly capable of acquiring ACAID and generating CD8$^+$ Tregs after intracameral injection of antigen. These studies put to rest the idea that ACAID was a deviation towards a Th2 response.

A Role for Chemokines in ACAID

During ACAID induction, MIP-2-producing F4/80$^+$ APCs appear first in the bloodstream and then in the spleen. MIP-2 is a murine functional analogue of human IL-8. In ACAID, the MIP-2 chemokine is critical to the recruitment of NKT cells and facilitating tolerance induction and was absolutely required for generation of the Ag specific CD8$^+$ Treg cells (23). ACAID also did not occur in mice that were deficient in CXCR2, the high affinity receptor for MIP-2. Thus, when the ACAID-inducing signal, F4/80$^+$ APC, leaves the eye, it begins the path toward tolerance induction by recruiting the types of cells that are needed to induce differentiation of T regulatory cells.

Another difference between immunogenic APC and tolerogenic APC is the chemokine receptor profile. Microgene array analyses performed in our laboratory showed that bone marrow derived F4/80$^+$ APC generated in cultures of bone marrow cells with L929 media expressed a unique chemokine receptor profile. BM–derived F4/80$^+$ APC treated with TGFβ2 and antigen were capable of inducing antigen specific tolerance in both naïve and previously sensitized mice (43–45). The in vitro generated tolerogenic BM–derived APCs do not express the critical chemokine receptor (CCR7) that is needed for cells to traffic to the T-cell areas of the spleen (43). This observation supports the postulate that the induction of tolerance occurs in the MZ rather than the T-cell areas of the spleen.

CONCLUSION

Thus, we propose that while the mature dendritic cell is the professional APC for the induction of an immune response, and presentation of antigen by an immature dendritic cell may lead to anergic T cells and peripheral tolerance, the tissue F4/80$^+$APC may be a professional APC for the induction of T regulatory cells.

The tolerogenic tissue APCs are found in tissues where immunosuppressive molecules reside and at least in the eye and gut express F4/80 and CD1d but not CCR7. Once in the spleen, the eye-derived F4/80$^+$ APCs remain in the marginal zone where they appear to encourage interaction of a unique group of cells. It seems that the cells within the aggregates create an immunosuppressive

microenvironment reminiscent of an immune privileged site. Like the induction of an immune response, there are complex interactions and of cell surface molecules leading to cross-talk and differentiation of T cells into Tregs. Moreover, $F4/80^+$ the APCs that may orchestrate the process of peripheral tolerance are not restricted to immune privileged sites since $F4/80^+$ APCs are required for the induction of oral tolerance. The possibility arises that APCs from other tissue sites rich in TGFβ may be equally capable of inducing Tregs and peripheral tolerance.

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4

The Role of Dendritic Cell Migration for the Induction of Immunity and the Maintenance of Tolerance

Manfred B. Lutz

Institute for Virology and Immunobiology, University of Wuerzburg, Wuerzburg, Germany

INTRODUCTION

Dendritic cells (DCs) and macrophages (Mphs) both belong to the group of professional antigen-presenting cells (APCs). Professional APCs endocytose extracellular material not only for nutritious or housekeeping reasons but also to process protein antigens and present them on MHC II molecules to T cells.

SIMILARITIES AND DIFFERENCES OF DENDRITIC CELLS TO MONOCYTES AND MACROPHAGES

To distinguish self from non-self, antigen DCs and Mphs are equipped with various pathogen recognition receptors to respond to microbial infections, such as Toll-like receptors, scavenger receptors, or indirectly though receptors binding complement- or antibody-trapped antigens. Both cell types also reside in almost all peripheral tissues as sentinels of the immune system (1,2).

Besides these similarities, there are some striking functional differences qualifying DCs as separate cell types and not as a subset of Mphs. DCs and Mphs use the same mechanisms to recognize and endocytose antigens; however, the total amount taken up differs significantly. While Mphs increase their phagocytic

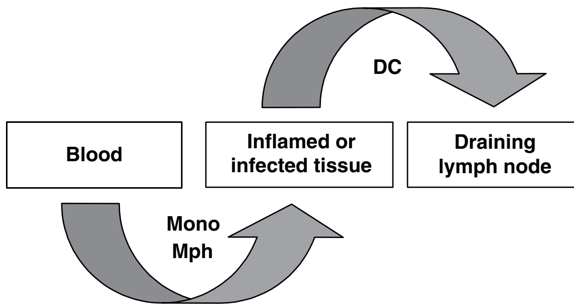

Figure 1 Different homing directions of monocytes/macrophages (Mono/Mph) versus DCs. During inflammatory or infectious processes in peripheral tissues, blood monocytes transmigrate through activated vascular endothelium into the tissue where they remain and exert their anti-microbial activities. Unlike, monocytes, tissue-resident DCs or derived from the immigrating DCs leave the infection site after uptake of foreign antigens and transport them to the draining lymph node.

activity after activation, DCs immediately downregulate phagocytosis. While activated Mphs locally start to exert numerous activities to further eliminate microbes, e.g., by the release of oxygen radicals or lysozyme, the tissue-resident DCs continuously disappear from this side (Fig. 1). DCs leave the site of infection/ inflammation, while Mphs rest there until the local bacterial load is eliminated. DCs emigrate via the afferent lymphatic to transport antigens to the T-cell areas of the lymph node, where they now can initiate a primary T-cell response (1,2).

SOLUBLE ANTIGEN TRANSPORT INTO THE LYMPH NODE (FIRST WAVE)

Recent evidence suggests, however, that the emigrating tissue-resident DCs might represent only the second wave of antigens reaching the draining lymph node for T-cell priming. Soluble fluorescence-labeled tracer antigens appeared in the lymph node of mice already 5–10 minutes after subcutaneous injection (3). There the tracer antigens were taken up by lymph node-resident DCs from the reticular conduit system and immediately processed and presented them to T cells (4). Reconstituted transgenic T cells expressing a T-cell receptor that could recognize the injected antigen were activated and divided but finally could not fully differentiate into effector cells, suggesting that further stimulation is required, which might also provide additional inflammation from the pathogenicity of antigen at the infection site (5). Possibly, the polarisation into T helper (Th)-1 and -2 effector cells requires a second wave or repetitive antigen presentation.

It is of note that the fluid-phase transport of antigens into the lymph node can occur only for small antigens (<80 kD) but not larger particles or whole bacteria. It is tempting to speculate whether resident Mphs within the inflamed tissue might phagocytose and process whole bacteria into smaller protein sized

antigens that are then exocytosed and delivered within the fluid phase to the lymph node. This transport of small soluble antigens might also be relevant for antigen delivery to B cells, as some tracer molecules can reach T- as well as B-cell areas, thereby finally enabling Th cell-dependent B-cell responses.

LANGERHANS CELL EMIGRATION FROM THE SKIN (SECOND WAVE)

Langerhans cells (LCs) reside in the epidermal layer of the skin and represent the best investigated DC type in vivo. In their immature or resting state they have the capacity to endocytose all type of antigens of all sizes by fluid phase macropinocytosis or receptor-mediated mechanisms (6). After microbial or inflammatory activation LCs undergo maturation, which is characterized by down-regulating the antigen uptake function. Then the enzymatic processing of antigens occurs in endosomal compartments followed by the presentation of high numbers of antigenic peptide/MHC I and II complexes at the LC surface. This maturation process is accompanied by the migration of LCs through the afferent lymphatics to the T-cell areas of the draining lymph node. LC migration through the lymphatics has been followed by painting the skin of mice with FITC or CFSE and the subsequent appearance of the LC in the lymph node (5,7). This active cellular migration involves numerous different molecular acivities by the LC and is therefore relatively slow (see below). About 16–24 hrs after fluid phase antigens entered the lymph node, a second wave of T-cell stimulation occurs by tissue-derived migratory DCs as shown for Langerhans cells and dermal DCs from the skin (5). Only now the T-cell activation (cell division, IL-2 production) reaches a level at which a strong immune response can evolve. It is unclear whether T-cell priming for larger antigens that cannot use the fluid-phase pathway is thereby less efficient. Presumably, a third or second wave, respectively, can occur by another DC population.

MONOCYTE-DERIVED DCs (THIRD WAVE)

Inflamed or infected tissues release cytokines and arachidonic acid metabolites that activate the vascular endothelium within the tissue. This activated endothelium enables the extravasation of neutrophils and later also of circulating monocytes into the inflamed tissue (8). Such monocytes can then differentiate into Mphs or DCs (9). It is unclear to date whether only certain predisposed subsets of $CD16^{+}$ versus $CD16^{-}$ monocytes is decisive for the differentiation into DCs or Mphs, respectively (10), or environmental factors can also influence this decision. While differentiated Mphs remain at the inflammatory/infected site, developing immature MoDCs will recognize and pick up the local antigens, which are then transported to the draining lymph node. Pathogen recognition will further induce migration and maturation of the MoDCs, which can then prime newly arriving naive T cells or perpetuate the stimulation of already primed but

not fully differentiated T cells as a third wave for small antigens or a second wave for larger antigens.

MECHANISMS OF DC MIGRATION

When peripheral tissues such as the skin are inflamed, infiltrated by microbes, or triggered by haptens, resident DCs start to emigrate via the afferent lymphatics to reach the draining lymph node (Fig. 2). This has been demonstrated by the transport of haptenized FITC, applied through skin painting, which was then transported by the skin DC populations (7).

After activation, epidermal LCs start to downregulate E-cadherin, the anchor receptor for binding to keratinocytes (11,12), and also α6-integrins, which enable the binding to the basement membrane (13) but upregulate CD44 (14). To cut a hole into the basement membrane to allow LC passage, the expression of the matrix metalloproteinases MMP2 and MMP9 is then required next (15). Endogenously released proinflammatory cytokines such as TNF-α or IL-1β, but also osteopontin, are major factors to promote the motility of DCs and thereby the migration process (16–19). Similarly, injections of LPS mobilized splenic DCs to migrate into the T-cell areas of the white pulp (20,21). Both endogenous and exogenous types of stimulators then induce other effector molecules, such as IL-16 (22), the multi drug resistance pump MDR-1, and chemokines (23), which further

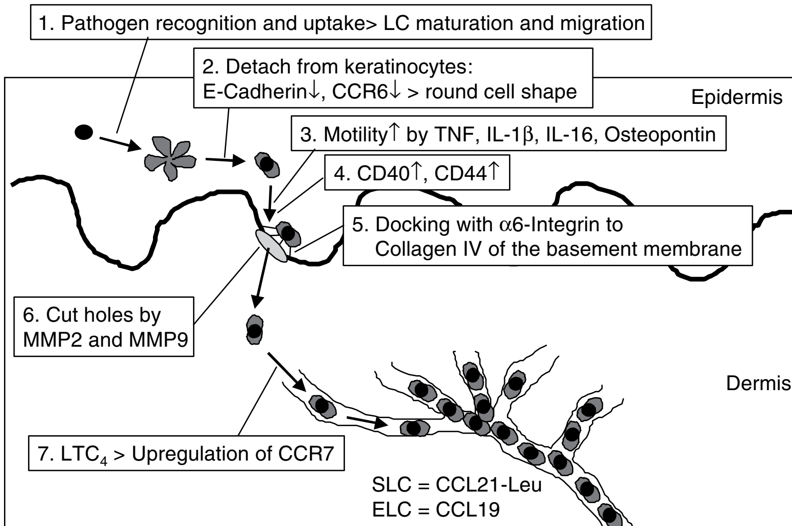

Figure 2 Molecular processes involved in Langerhans cell emigration from the skin. Inflammaory or pathogen-induced maturation induces a cascade of events which enable the cells to leave the epidermis and find their way through the lymphatics and finally to the draining lymph node.

promote LC emigration from the skin. Simultaneously, the chemokine receptor CCR7 is induced to guide the LCs through the afferent lymphatics that express the ELC/CCL19 and SLC/CCL21-leu chemokine and into the lymph node by the ELC/CCL19 and SLC/CCL21-ser chemokines (24–27).

MIGRATION OF DCs CELLS IN THE STEADY STATE FOR TOLERANCE INDUCTION

Apart from the induced DC emigration from the peripheral tissue after infection or inflammation, spontaneous migration of DCs can also be observed under steady-state conditions. In peripheral organs, as well as in secondary lymphoid organs, a turnover rate of the residing DC populations can be observed. Also, during homeostasis DCs leave tissues via the lymphatics and can there be detected as so-called "veiled cells." Cannulated, afferent lymph studied in sheep (28), rats (29,30), pigs (31), and humans (32) is enriched of veiled cells, which represent DCs on their way to the lymph node. LCs migrating out of the skin into the peripheral lymph nodes were calculated to have a half-life of about one month under homeostatic conditions (33,34). The turnover of DCs in secondary lymphoid organs is even more rapid with about 3–5 days (35–37).

A closer look into the cytoplasm of veiled cells shows that they are not traveling "empty" but carry antigens or apoptotic cells derived from the tissue from which they were originating. Many years ago, epidermal LCs were shown to contain melanosomes when emigrating from healthy skin (38), and, more recently, it was shown that this transport of melanosomes decreases in the absence of TGF-β1-dependent LCs (39). Mucosal LCs have also been reported to capture apoptotic epithelial cells in the vagina and cervix (40). In the rat afferent lymph draining from the gut, a subpopulation of DCs contains apoptotic material from their sentinel tissue, which is brought into the mesenteric lymph node (41). Self antigens are transported to the draining lymph node from the pancreas (42) and stomach (43). Also, cells undergoing apoptosis and entering the lymph node through the lymphatics can be captured by lymph node resident DCs for self-antigen presentation (44,45). As no foreign antigens are present for steady state migratory DCs, it is strongly suggested that such DCs induce tolerance to tissue antigens by direct presentation to $CD4^+$ T cells (46,47) or by cross-presentation to $CD8^+$ T cells (42).

IS MATURATION REQUIRED FOR MIGRATION OF DCs?

The morphological and phenotypical differences between tissue resident DCs and migrating DCs are dramatic and similar to those occurring upon DC maturation (Fig. 2). Therefore some degree of maturation has been postulated also for mediating homeostatic antigen transport (23,48). Only DCs that express CCR7 to enable their homing into the T-cell areas of the lymph node (24). On murine bone marrow-derived DCs and peripheral lymph node DCs the expression of CCR7 is

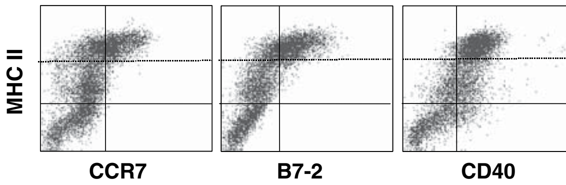

Figure 3 Expression of CCR7 is linked to DC maturation. DCs were generated from mouse bone marrow with GM-CSF until day 8 and then stained for the indicated markers. Spontaneously matured MHC IIhigh, B7-2high, and CD40 high expressing cells within the culture were the only ones that also stained with an ELC-IgG fusion protein, detecting CCR7 on DCs.

strictly linked to an MHC IIhigh, B7-2high, and CD40high phenotype (Fig. 3 and our unpublished data).

On their way, also in the steady state, antigen processing has to occur, followed by loading of MHC II and I molecules and their transport to the surface together with costimulatory molecules. For antigen processing and tolerogenic cross-presentation of apoptotic material, at least some maturation of DCs seems to be required (44,49). We have shown that TNF-α is inducing an incomplete maturation of DCs, and that such semi-mature DCs could still act in tolerogenic manner, as they were able to protect mice from autoimmunity (47,50). Similarly, pulmonary DCs pulsed with antigen for tolerance induction are "mature" DCs after reaching the draining lymph node (46). Thus, tolerance induction by migrating DCs in mice requires at least partial maturation.

For human DCs, it has been described that, under chronic inflammatory conditions, skin-derived DCs accumulate in the draining lymph nodes, but appear rather immature (51). The question remains: Is a specific degree of maturation required, and/or is there a specific signal for DCs to start this "spontaneous" migration?

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5

The Activation Status of Dendritic Cells Is Crucial for Decision Making on Tolerance Versus Immunity

Karsten Mahnke and Alexander H. Enk
Department of Dermatology, University of Heidelberg, Heidelberg, Germany

INTRODUCTION

Dendritic cells (DCs) are distributed throughout the body in virtually all tissues, where they constantly sample the environment. Eventually, DCs migrate towards local lymph nodes and present major histocompatibility complex (MHC)–peptide complexes to T cells (1). Here, presentation either leads to activation of the T cell, or results in anergy of the respective T cells, depending on the circumstances.

DCs have been characterized as potent immunostimulatory cells in numerous experimental settings (2). However, in these scenarios, pathogens invaded the tissue, resulting in activation of the DCs and induction of immune responses (3–6). The opposite takes place under steady state conditions, i.e., in the absence of pathogens or tissue disruption. Here, the DCs remain in a non-activated, immature status in the tissue and sample their environment by taking up proteins and cell-derived detritus. Upon antigen presentation in the lymph node, T-cell proliferation is curbed by the means of regulatory T cells and/or by deletion of T effector cells. Thus, under "pathogen-free" conditions, i.e., when DCs remain in a non-activated state, presentation of endocytosed "self"-antigens leads to tolerance (7,8).

In this series of events, ranging from uptake of antigens to presentation of MHC-peptide complexes to T cells, the uptake of antigens is a first crucial step. The quality of the antigen, as well as under which circumstances the respective

antigen has been taken up by the DC, is of importance. For instance, the DCs have to distinguish between pathogen-derived "foreign" antigens, harmless "foreign" antigens or "self" derived antigens, respectively. Accordingly, the "quality" of the antigen provides further information on whether immunity or tolerance should be accomplished (9).

Within the tissue, DCs possess the capacity to take up different kinds of antigens by different means, i.e., pinocytosis or phagocytosis. Pinocytosis is the uptake of solubilized fluid-phase components and is the main mechanism used by immature DCs to acquire antigens. This uptake allows DCs to sample large amounts of their tissue environment but has the disadvantage that substances are randomly picked from their vicinity and later stages of endosome sorting must ensure that respective MHC-peptide complexes will be generated (10,11).

Receptor-mediated endocytosis, however, employs receptors that are highly specific for ligands and thus provides a means to the cells to control which antigens to take up. Moreover, the guided intracellular targeting of the receptor–ligand complexes enables the cells to determine in which intracellular compartment the ligand will be unloaded for further processing, i.e., resulting in production of antigenic peptides or complete proteolysis. Furthermore, the interaction of endocytic receptors with signaling pathways in DCs provides information for the induction of an appropriate immune response, i.e., tolerance or immunity. Therefore, receptor-mediated endocytosis displays a tool to guide the selective antigen uptake and processing and may be involved in selection of pathways that either lead to induction of immunity or to induction of tolerance (12).

ANTIGEN UPTAKE BY DCs

The Quality of the Antigen Determines the Response

The influence of the intracellular targeting upon antigen presentation is underlined by observations of the targeting of the tumor antigen MUC-1. This antigen is covered with mannose and galactose residues and binds clearly to C-type lectins, in particular to the MMR and/or to the galactose-N-galactosamine-specific lectin (MGL) (13,14). Upon binding to these receptors, MUC-1 is endocytosed properly, but the further intracellular targeting to lysosomal compartments is impaired, leading to poor antigen presentation and to inferior T-cell responses. If the sugar residues from the MUC antigen are removed, the intracellular targeting to lysosomal compartments is restored (15). Most likely, this behavior is explained by the multivalent binding of many C-type receptors, whereby the proper targeting is prevented by those multi-receptor antigen aggregates.

Indeed there are examples of pathogens that are highly specialized to utilizing certain C-type lectins for escape (as extensively reviewed by Engering) (16). For example, the HIV virus enters $CD4^+$ T cells using the lectin DC-SIGN, and impairs its intracellular targeting to lysosomes. Therefore, the virus remains active, hides within the DCs, and uses the DCs as a transport vehicle towards

lymphatic organs. Once the infected DC reaches the lymph node and gets into contact with $CD4^+$ T cells, the virus is able to return to the surface and to infect the adjacent T cells (17). Similar mechanisms have been observed with Mycobacteria (18), which enter DCs, but are able to escape the antigen-processing machinery. In these examples of tolerance (or at least ignorance) towards certain pathogens, the respective lectin-like receptors are "corrupted" by the binding microorganisms and play a merely passive role, but some lectins possess intrinsic signaling capacity to downregulate immune responses by different means.

Signaling by Antigen Receptors

In addition to those different intracellular routing patterns of the C-type lectins, direct signaling may also be involved in conveying tolerogenic signals. The DC immunoreceptor (DCIR) (19), for example, bears an immunoreceptor tyrosine-based inhibition motif (ITIM) in its intracellular domain. ITIM has been previously analyzed in the inhibitory FcR-IIB and is thought to prevent calcium signaling and, henceforth, activation of effective antigen presentation by the DCs. In contrast, the MMR does not contain any ITIM, but is capable of interfering with Toll-like receptor (TLR) signaling, which normally activates DCs upon binding of microbes. In those experiments, it has been shown that simultaneous engagement of the MMR and TLRs inhibits production of IL-12, which is normally triggered after binding of bacteria derived lipoproteins (20). This lack of IL-12 production results in immune deviation and favors development of TH-2 like immune responses that allows prolonged survival of microorganisms.

C-TYPE LECTINS: DEC-205 AND MMR AS PROTOTYPE RECEPTORS WITH DIFFERENT PATHWAYS

Recently, many different C-type lectins have been characterized in DCs, ranging from molecules containing single carbohydrate recognition domains (CRDs), to deca-lectins that comprise 10 CRDs (21). The presence of CRDs implies that glycoproteins are the most likely candidate. The expression of different CRDs within one receptor allows the defined binding of specific sugars. For example, the mannose receptor, MMR (CD206) (22), but not DC-SIGN (CD209) (23) or Blood-DC-Antigen–2 (BDCA-2) (24), binds to end-standing single mannose residues, and DC-SIGN binds to mannose only when complexed with other sugar moieties.

Most of the Lectin receptors mediate endocytosis by leucine- or tyrosine-based internalization motives; however, further amino acid sequences may determine the internalization route and, finally, the fate of the receptor ligand complexes. The prototype lectin receptor, the macrophage mannose receptor (MMR), is internalized into coated pits and transports its ligands to early endosomes. Here, separation of the ligand–receptor complexes occurs, and the MMR recycles back to the cell surface. In contrast, its sister molecule, DEC-205

(CD205), defines a novel pathway by recycling through late endosomal, MHC class II positive compartments (25). Guided by 3 acidic amino acids (EDE-sequence), the DEC-205 receptors takes ligands to LAMP$^+$, MHC-class II$^+$ late endosomal compartments in DCs, that resembles MIICs (26). These compartments were extensively studied by Mellman et al. and play a pivotal role in loading antigenic peptides onto freshly generated MHC class II molecules. Accordingly, DEC-205–driven antigen presentation resulted in up to a 100-fold enhanced T-cell stimulation as compared to antigens that were endocytosed by the MMR. This intracellular targeting of DEC-205 defines a novel pathway of antigen presentation since the receptor guides the ligand directly into the antigen presentation compartment, and perhaps the different targeting routes of the different lectins may contribute to whether tolerance or immunity is accomplished.

THE ACTIVATION STATUS OF DCs INFLUENCES THE QUALITY OF THE IMMUNE RESPONSE

Induction of Tolerance by DCs in the Steady State

Different attempts have been made to analyze the resulting T-cell answers upon presentation of antigens by ex vivo isolated DCs, cell lines, or differentiated DCs, respectively. These in vitro manipulations result in a phenotype that resembles DCs in an activated status, and is far from in vivo situations where antigen uptake normally takes place under steady state conditions. In an attempt to mimic the in vivo situation and to study the contribution of antigen receptors to the development of T-cell answers, we employed antibody targeting to load DCs in vivo under steady state conditions (27–29). In these investigations, a model antigen OVA and hen egg lysozyme (HEL) were covalently linked to anti-DEC-205 antibodies and injected into mice. This resulted in specific antigen loading of the DCs in the lymph node and to presentation of those antigens to OVA and HEL specific T cells, respectively. The analysis of the induced immune response after targeting DCs in the steady state revealed that tolerance was induced, and two possible mechanisms were obvious: Hawiger et al. could demonstrate T-cell deletion (28), whereas our own results point towards induction of regulatory T cells (Tregs) (29). Both mechanisms are not mutually exclusive, since induction of Tregs was observed approximately eight days after loading the DCs in vivo, and T-cell deletion in the "HEL system" was recorded three weeks after the original challenge of the DCs. Possibly both pathways, i.e., induction of Tregs as well as deletion, may interact with each other, with the induction of Tregs being a very early response to dampen activation of T cells immediately and locally contained, followed by the deletion as a final means to get rid of the T cells and to ensure long lasting tolerance towards that respective antigen.

The exact means are not clear yet; however, both mechanisms are further corroborated by in vitro experiments. Direct induction of apoptosis, for example, has been demonstrated in the murine system (30). It was shown that a certain

subtype of DCs expresses the Fas ligand, enabling these DCs to kill Fas-bearing activated T cells. However, those effects did not depend on the activations status of the DCs. Rather, a certain subtype of DCs seemed to be involved in this tolerogenic action. Interestingly, those DC subsets also express the DEC-205 receptor, and it is conceivable that DEC-205$^+$ DCs are prone to induce tolerance rather than immunity. Lu et al. (31) show that DEC-205$^+$ liver DCs are poor T-cell stimulators and induce T cells that produce the anti-inflammatory cytokine IL-10. In addition, a subset of DEC-205$^+$/CD8$^+$ DCs has been identified in mouse spleen, that is able to curb proliferation of CD8$^+$ T cells in vitro by inducing TH2-like cytokines (32). So, even if the functional aspects of DEC-205–mediated antigen presentation to the tolerance induction remains elusive, DEC-205 expression might serve as a suitable marker to characterize tolerogenic DCs (33,34). In addition to killing T cells after antigen presentation, DCs are also capable of inducing T cells with regulatory properties. For example, CD4$^+$/CD25$^+$ Tregs can be induced by stimulation of T cells with immature DCs in vitro (35), and injection of immature DCs lead to induction of CD8$^+$ T cells with regulatory properties in cancer patients (36,37).

Induction of Immunity by Activated DCs

In consequence of the paradigm stating that immature DCs induce tolerance, it is conceivable that mature and/or activated DCs induce immunity. This conclusion has been proven several times. Hawiger et al. (28) as well as Mahnke et al. (29) reported that they could obliterate the induction of Tregs and the deletion of T cells after DEC targeting if DC-activating stimuli such as anti-CD40 antibodies were administered simultaneously.

In these experiments, the DCs were activated by engaging the CD40 receptor via specific antibodies prior to injection of the anti-DEC-antigen conjugates, and presentation of the respective model antigens (HEL or OVA respectively) lead to vigorous T cell proliferation without the induction of Tregs.

Using the same method of antibody-mediated targeting to DCs in vivo as described for tolerization purposes, our own recent results further substantiate the connection that the activation status of DCs is crucial for the outcome of an immune response.

In an attempt to induce protective anti-tumor immunity, the melanoma antigen tyrosinase-related protein 2 (TRP2) was coupled to anti-DEC antibodies and injected into mice. Simultaneously, a DC-activating stimulus by injection of CpG was administered. After two consecutive injections of these anti-DEC–tumor antigen conjugates, mice were challenged for tumor growth by intravenous injection of the melanoma cell line B16. After two weeks, metastases in the lungs of the mice were counted. Our results clearly show that mice were protected from tumor growth after two immunizations with antibody–tumor antigen conjugates when injected together with an activating stimulus, i.e., CpG (38). This protection was lost when CpG was omitted from the immunization procedure, indicating that activation of the DC is mandatory for effective induction of an immune response.

CONCLUDING REMARKS

DCs have originally been characterized by their potent T-cell stimulatory properties, but recent results show that they are also able to downregulate immune responses by means of deletional tolerance or the induction of different types of regulatory T cells. Although the mechanism by which DCs accomplish this is not entirely clear yet, there is evidence that the final outcome of an immune response (i.e., immunity versus tolerance) is influenced by the status of DCs as well as the quality of the antigen itself; i.e., presentation of antigen by activated DCs induced long-lasting T-cell proliferation and immunity, whereas DCs in the steady state induce regulatory T cells or delete antigen-specific T cells, respectively. Taken together, these results highlight a novel mechanism that provides a means to tolerize against antigens present in the periphery of the body. In the absence of inflammation, tissue-residing DCs constantly sample their vicinity and take up numerous antigens. Those antigens are mainly derived from apoptotic cells and harmless environmental antigens, respectively (39,40). Upon uptake and presentation of those antigens, tolerance by the means of T-cell deletion and/or induction of Tregs is accomplished. This is beneficial in two ways: potentially self-reactive T cells that escaped the thymic selection will be deleted from the periphery of the body, and useless immune responses to otherwise harmless environmental antigens are prevented. In case pathogens are present, DCs become activated through pattern recognition receptors (41) and/or Toll like receptors, leading to upregulation of T-cell stimulating molecules (such as B7-1, B7-2, CD40, MHC-II) and to induction of a vigorous T-cell response towards the respective antigen.

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6

Distribution of Antigen-Presenting Cells in the Eye

Paul G. McMenamin, Season Yeung, and Serge Camelo
School of Anatomy and Human Biology, University of Western Australia, Crawley, Western Australia, Australia

INTRODUCTION

A number of vision-threatening eye conditions, for example, corneal transplant rejection, infections, response to intraocular tumors, choroidal neovascularization, and uveitis, have an immunological or inflammatory basis. One of the hurdles in the treatment and therapy of these conditions is that their immunopathogenesis is incompletely understood. Antigens (Ags) derived from donor corneal grafts, the lens, infectious agents, damaged retina, or degenerating/growing tumors in the eye can all potentially generate immune responses, either of an innate or adaptive type, and in the latter case these may be of an immunogenic or tolerogenic nature. Antigen presenting cells (APCs) are crucially positioned in the pathways that determine the nature and types of immune response. Therefore, the nature, function and distribution of APCs in the eye in relation to sites of exposure to potential pathogenic agents will likely be important determining factors in the resultant immune response. For the purposes of this review the term "APC(s)" is used inclusively for both macrophages and dendritic cells (DCs). Other APCs, such as B cells (which seldom occur in the normal eye), or parenchymal/non-bone marrow-derived cells that can potentially assume an APC function in abnormally disrupted states, are not the focus of this review.

Some 10 to 15 years ago it may have been pertinent to ask if there were any APCs in the eye; however, we can now be reasonably confident that this issue has been resolved in the affirmative. Therefore this review gives the reader a brief synopsis of the state of knowledge regarding the distribution of APCs in a broad range of tissue sites in and around the normal eye. Examples from a variety of species are discussed, as it seems unlikely that the evolutionary and functional advantage underlying the distribution pattern of cell types that perform such crucial roles in both adaptive and innate immune responses will vary dramatically between mammalian species. It seems self-evident that most species of mammals are likely faced with similar potential external environmental factors, be it physical injury or infection by potential pathogens. Therefore one would imagine that most species for which vision is an important sensory modality would require similar homeostatic mechanisms, including immunological, that aid in maintaining ocular transparency (1).

An understanding of the complex three-dimensional anatomy and structure of the eye together with the advantages offered by various technical approaches, such as immunohistochemical staining of tissue wholemounts, has greatly advanced the discovery and description of these cells. The importance of these issues is highlighted where appropriate. Furthermore, the functional implications of the presence of APCs in different parts of the eye and their possible role in afferent and efferent arms of ocular immune responses are discussed.

WHAT IS AN APC? THE DIFFERING ROLES OF MACROPHAGES AND DCs IN IMMUNE RESPONSES

It is important to establish early in this review that dendritic cells (DCs) are considered to have widely different roles from macrophages in immune responses. The former are a heterogeneous group of potent APCs that initiate primary and anamnestic immune responses. They have a complex life-cycle in which their occupancy within peripheral tissues and their ability to migrate to secondary lymphoid organs seems ideally adapted to surveillance or sentinel function. They play a pivotal role in the induction of central and peripheral tolerance and in regulation of T-cell responses in adaptive immune responses (2,3) but more recently they are being recognized as regulators of innate immune responses (4), which has resulted in a further dramatic surge in enquiry about the biology of these cells.

Life Cycle and Function of DCs

Bone marrow-derived DC precursors migrate into peripheral tissues (via the blood) where as "immature" DCs they are characterized by dendritic morphology, constitutive MHC class II expression, low co-stimulatory molecule expression, and network arrangements. Functionally, they are specialized for antigen

trapping/processing and possess weak immunostimulatory capacity (2). Upon Ag contact or following tissue perturbations (necrosis, inflammation, injury) they migrate via lymphatic vessels to T-cell dependent areas of local lymphoid tissues. Upregulation of CCR7 on DC aids in directing their migration, guided by localized expression of chemokines and other related molecules in secondary lymphoid organs (5). DCs mature in the T cell zone, a process that takes around 12 hours and lasts for 2 to 3 days (5). Here they gain potent antigen-presenting capabilities and present Ag/peptides within the groove of the MHC class II molecule to naïve T cells, resulting in either activation, tolerance, and peripheral deletion, or apoptosis (6). There is currently a lively debate that is reassessing the role of peripheral tissue-derived DC and the function of the DC subsets (up to 6) present in all secondary lymphoid organs (3).

MACROPHAGES

Macrophages are a versatile group of cells of the mononuclear phagocyte system that are intimately involved in all aspects of immune responses and inflammation. They comprise a heterogeneous population of resident and recruited cells in all tissues (7). Resident tissue macrophages (RTMs) are more ubiquitously distributed than DCs in non-lymphoid tissues and display responsiveness to many exogenous and endogenous stimuli including characteristic phagocytic and endocytic activity. A paradigm of macrophage heterogeneity in terms of phenotype and activation is now emerging (for review, see Ref. 7).

1. *Innate activation* includes responsiveness to microbial invasion via pathogen recognition receptors [e.g., Toll-like receptors (TLRs)], CD14-LPS binding protein and non-opsonic receptors leading to production of pro-inflammatory cytokines (IFN-γ, reactive oxygen species, nitric oxide) followed by a regulated anti-inflammatory response. Activation of macrophages via scavenger receptors or mannose receptors promotes phagocytic activity.
2. *Humoral activation* and phagocytosis are mediated via Fc and complement receptors and lead to cytolytic activity and production of pro- or anti-inflammatory cytokines.
3. *Classical activation* is mediated by IFN-γ and its receptor on macrophages together with a microbial trigger (e.g., LPS) and leads to upregulation of MHC class II and production of pro-inflammatory cytokines such as IL-6, TNF, IL-1, and nitric oxide burst. This form of response is crucial in microbiocidal activity, cellular immunity, delayed type hypersensitivity (DTH) responses, and can result in local tissue damage.
4. *Alternative activation* is mediated by IL-4 and IL-13 acting through a common receptor chain (IL-4Rα). It leads to upregulation of MHC class II and mannose receptors, increased phagocytic activity, and is

important in humoral immunity, allergic responses, anti-parasitic responses, and tissue repair.

5. *Innate/acquired deactivation* can be generated following the uptake of apoptotic cells or lysosomal storage of host molecules and may be modulated by a range of receptors. The response leads to downregulation of MHC class II and production of anti-inflammatory cytokines (TGF-β and IL-10) and PGE_2.

Resident tissue macrophages supplemented by newly recruited monocyte-macrophages act as important sources of cytokines in normal tissues, and their phagocytic role in wound repair (fibrin dissolution, removal of dead tissue), fibroblast recruitment, growth, and remodelling, including neovascularisation, aid in the return to normal function post-injury or inflammation (7). Macrophages are efficient stimulators of primed T cells but unlike DCs are poor initiators of primary immune responses due to their lack of expression co-stimulatory molecules that are a prominent characteristic of DC phenotype and DC-TCR interactions. Macrophages appear to be less migratory than DCs and although they have been reported to migrate to draining lymph nodes, they do not enter efferent lymph and thus the thoracic duct (8). The dichotomous role of DCs and RTMs in immune responses is likely the underlying reason why the distribution of DCs and RTMs, while sometimes being closely linked, do differ noticeably in some ocular tissues (e.g., retina). Such differences may underlie their contributions to immune and non-immune mediated ocular diseases.

CORNEA-LIMBUS

A vivid reminder of the necessity to understand immune responses in the cornea is the rate of corneal graft rejection. Despite human corneal transplants being the most frequently performed solid organ/tissue transplant (45,000 in the United States), clinicians and laboratory investigators alike struggle to find a means of assuring long-term acceptance, especially of high risk grafts (9). Although the two-year survival rate of human corneal grafts is over 86% to 90%, it would undoubtedly be considerably lower in the absence of immunosuppressive therapy. Failure to use such therapy in animal models of corneal transplantation (using non-syngeneic hosts and donors) results in an approximately 50% rejection rate (9,10).

In light of the importance of potential "passenger cells" in donor corneal grafts and the role of host resident immune cells such as RTMs and DCs close to the edge of the host bed, there has been considerable interest in the normal distribution pattern of these cells in the cornea. There is a considerable body of early literature indicating MHC class II$^+$ DCs or Langerhans cells (LCs, analogous to the populations of epidermal LC of the skin) are present within the limbal and peripheral corneal epithelium and display a centripetal density gradient (vide infra) (11,12). This pattern is particularly evident when one takes a "plan" view of the entire cornea or wholemounts of corneal and limbal epithelium

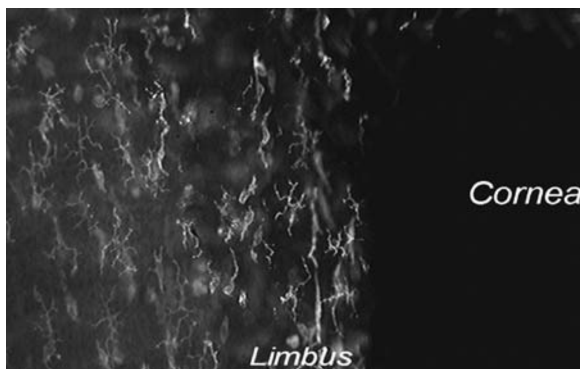

Figure 1 Rat corneal-limbal wholemount stained with anti-MHC class II mAb and visualized by epifluorescence microscopy. Note the high density of cells in the limbal region and the decreased density in the peripheral cornea. The highly dendriform cells (closest and most in focus) are the intraepithelial DCs (or LCs).

(readily removed for staining from EDTA pre-treated corneas) (Fig. 1). Many early studies utilized a variety of histochemical (ATPase), immunohistochemical markers (predominantly anti-MHC class II mAbs) and conventional transmission electron microscopy (in which Birbeck granules are taken as a definitive marker of LC) to map out DC populations in the cornea (13).

Central Cornea

It has been accepted for some time that the central cornea was devoid of MHC class II$^+$ cells (or Ia$^+$ in rodents) (12–15); however, they were recognized as being present in the peripheral epithelium and stroma of the murine cornea (14,15). Identification of ATPase$^+$ dendriform cells in the peripheral and pericentral murine cornea (13,14,16) appeared to confirm the presence of DCs in these zones. This concept of a centripetal gradient in density corneal epithelial LCs was found to be true of a number of species (Table 1).

The view that the normal central cornea was devoid of bone marrow-derived cells was offered as part explanation for the lack of immunogenicity of corneal grafts. There were isolated reports of the presence of rare MHC class II$^+$ DCs/LCs in the central corneal epithelium of human and some non-human species (see reviews, 17,18). Marked migration of epithelial LCs into the central cornea can be induced by various stimuli such as suturing and electrocautery (18). There were only isolated reports of MHC class II$^+$ cells in the corneal stroma and almost no evidence that they extended any more centrally than the peripheral cornea. These observations were held to be true for many years and helped form a central tenet underpinning the basis of the immune-privileged nature of the cornea, namely that there was a lack of passenger cells in a donor cornea (9,10). Indeed, in support of this hypothesis it was shown many years ago by Streilein and

Table 1 Surface Density of Langerhans Cells in Normal Ocular Surface Epithelium in Various Species (cells/mm^2)

	Species			
Region	Human	Guinea pig	Mouse	Rat
Conjunctiva	200–400	200–300	100–150	200–400
Limbus	150–350	200–300	150–200	200–400
Peripheral cornea	75–150	25–50	50–100	25–50
Pericentral cornea	25–50	None	25–50	None
Central cornea	None	None	None	None

Source: From Ref. 13.

Neiderkorn that if one induced the migration of these LCs from the limbus to the central cornea (vide supra), the success rate of subsequent transplantation of these corneas into naïve hosts dropped dramatically (11).

Equally, the reason behind the lower success of human corneal graft acceptance in hosts with vessels encroaching the surgical margin or evidence of inflammation was thought to be due to the presence of host LCs or DCs at the host–graft interface and their subsequent exposure to donor corneal antigens (9).

These were the widely held views until recently when two independent groups re-investigated the issue. In the first instance, distinct populations of CD11c$^+$ but MHC class II$^-$ DC were noted in the central corneal epithelium (19). In a further study expanding on these observations the same group detailed a population of myeloid DCs (CD45$^+$, CD11c$^+$, CD11b$^+$, MHC class II$^+$, CD80$^-$, and CD86$^-$) in the peripheral stroma of normal mouse cornea, but these cells appeared to lack MHC class II expression in the central stroma (20,21). In accordance with many older studies, these authors noted the density of immunopositive cells in the anterior stroma decreased from the periphery towards the centre (23). In contrast, around the same time an independent group identified a population of cells in the posterior corneal stroma that were CD45$^+$and CD11b$^+$ co-expressed F4/80, but were CD11c$^-$ and MHC class II$^-$, suggesting they were of the macrophage lineage (22). These authors failed to identify CD11c$^+$ cells (putative DCs) in the central cornea and highlighted the difficulties of fixation in the outcomes of immunostaining results in whole corneas.

Hamrah et al. (19–21) also identified a subpopulation of immunopositive cells within the posterior murine stroma with the phenotypic profile of macrophages (CD45$^+$, CD14$^+$, CD11b$^+$ CD11c$^-$, and MHC class II$^-$ cells). The findings were not strain specific as they were replicated in three different strains of mice (BALB/c, C57BL/6, and C3H) (22,23). More recently, Nakamura et al. (23) with the aid of eGFP transgenic bone marrow chimeric mice were able to investigate using direct in vivo observation the turnover of eGFP immune cells in the cornea, which they suggested was between 2–6 months.

Are these reports of macrophages and DCs in the central cornea of the mouse relevant to other mammals including humans? Moderate densities of MHC class

II$^+$ or ATPase$^+$ cells have been detected in the peripheral corneal epithelium of rat (13,16,24), guinea pig (13,16), and cattle (25). These cells have only rarely been reported in the central epithelium and stroma of normal rat cornea (26). In humans, occasional CD45$^+$ HLA-DR$^+$ (MHC class II$^+$) cells have been detected within the peripheral corneal epithelium (13,27–33) and only a few studies have reported the presence of isolated, rare or occasional MHC class II$^+$ cells in the normal central corneal epithelium (28,29,31). The density of these cells in the central fetal and infant cornea has been shown to be significantly higher than that of the adult cornea (34–36). As for the stroma in the human cornea, there have been occasional reports of CD45$^+$ HLA-DR$^+$ cells in the anterior one-third, which display a centripetal density gradient similar to that described in other species (27,28,30–32).

In other non-mammalian species a population of cells with the histochemical (ATPase$^+$) and some partial immunophenotypic profile (markers are limited for many species) of DC and LC have been detected in the peripheral corneal epithelium of frogs [*Rana pipiens* (37)], chicken [*Gallus gallus* (38)], and Atlantic salmon [*Salmo salar* (39)]. Thus it appears that there is still an overwhelming trend in the literature towards there being DC-like cells in the peripheral and paracentral cornea but few in the central cornea. However, with the limited availability of reagents and the technical difficulties in staining and detecting these cells in corneal wholemounts of thicker corneas (sections being of very limited value for such scantly distributed populations), it is possible that the results in mice may yet prove to be of wider relevance in mammals and non-mammalian vertebrates. Supportive evidence from other species is eagerly awaited.

The distribution of RTMs in the cornea of other mammals and non-mammalian vertebrates is less clear as these have generally not been the focus of previous investigations.

If indeed DC and/or macrophage populations do exist in the central cornea of most mammals, then obviously they have largely evaded detection by many investigators. The technical reasons for this apparent evasion may include:

- difficulties such as visualizing complex shaped cells in conventional sections
- ensuring sufficient penetration of mAbs into the dense connective tissue of the cornea
- the notoriously temperamental nature of staining with some mAbs (such as CD14 and CD11c)
- differences in fixation protocols affecting immunophenotypic staining
- paucity of phenotypic markers in species other than mice

Limbus

Within the limbus and conjunctiva, putative DCs are abundant both in the epithelium (classical LC-like cells) and in the subepithelial connective tissue (Fig. 2A–C) of a number of species (13,16,40,41). The subepithelial populations co-exist alongside typical RTM which display a perivascular distribution (42).

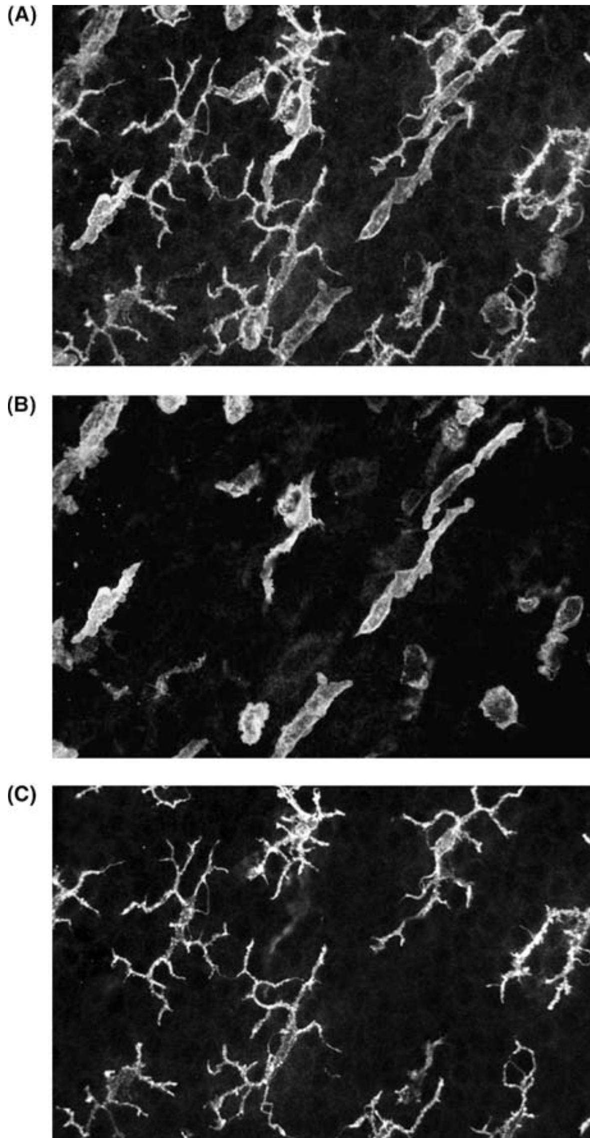

Figure 2 Confocal images of rat corneal-limbal wholemount stained with anti-MHC class II mAb as shown in (A) illustrates the entire confocal Z-series (optical sections), which includes the epithelium, sub-epithelial tissue and stroma. Note the large density of MHC class II+ cells. (B) Only the deeper layers of the Z-series are included which reveals that cells in the sub-epithelial connective tissue are an irregular-bipolar shape with a longitudinal orientation (likely following the adjacent vessels). (C) The upper layers of the Z-series (epithelial region of the limbus) reveal the highly dendriform nature of the intra-epithelial DCs (LCs) when compared to the non-epithelial neighbors in (B).

The presence of a rich network of HLA class II+ or ATPase+ cells in the human limbus has been confirmed by numerous studies (31,32,35,43,44). The majority of these cells are located in the limbal epithelium (45), although they are also present in the limbal stroma (31). Likewise, HLA-DR+ and CD1a+ cells have been detected in the normal human conjunctival epithelium (29,33,46–49) and in the supporting connective tissue (33,46). There are fewer studies of the macrophages in this connective tissue but they are present in large numbers in a manner similar to other non-ocular connective tissues underlying epithelia exposed to environmental antigenic challenges (42,50).

What does the phenotype, distribution, and morphology of DCs and RTMs tell us about the function of cells in the cornea, limbus, and conjunctiva? The epithelial DC/LC networks with their processes interposed between the epithelial cells appear optimal for Ag surveillance and trapping function at this environment–tissue interface. Figure 2 illustrates the difference in morphology between intraepithelial DCs (Fig. 2C) and those immediately beneath the epithelium (Fig. 2B). The pattern and morphology resemble similar populations in the epidermis of the skin and other mucosa such as the respiratory epithelium and gut (51–53). Recent data have begun to elaborate the mechanisms of Ag capture by intestinal DCs that extend processes between epithelial cells to the gut lumen where they trap Ags and subsequently traffic to local lymph nodes (54). The similarity in morphology, distribution and immature phenotype of DCs in the limbus and conjunctiva to the skin and other mucosal surfaces is hardly surprising in light of the functional similarities (55).

Evidence has recently emerged of the capacity of corneal DCs to migrate to local lymph nodes (20,21) but there has been less attention paid to the question of their Ag-trapping ability. Work in our laboratory is beginning to address this issue and has revealed that while corneal and limbal macrophages have the capability of capturing Ag when it is encountered beneath the epithelium (Fig. 3), it does not appear, from our experiments, that DCs play a major role in Ag uptake in the cornea. To date we have been unable to detect Ag uptake from the ocular surface in the absence of a breach in the epithelium (unpublished data).

THE UVEAL TRACT (IRIS, CILIARY BODY, CHOROID)

In recent years it has become evident that there are a plethora of local microenvironmental factors in the eye, including expression of FasL (56); high concentrations of TGFβ and immunomodulatory neuropeptides (αMSH, VIP, and CGRP) in the aqueous humour; and the expression by the iris pigment epithelium of molecules, such as CD86 and defensins, that contribute towards maintaining ocular immune privilege or suppress innate and adaptive immune responses (1,57). DCs are of course central to the regulation of the adaptive immune responses, and while they were once thought to be absent from the uveal tissues, this is no longer the accepted view. Numerous immunomorphological and functional studies (58–65) have established that there are extensive networks of MHC class II+ DC and RTM in the uveal tract

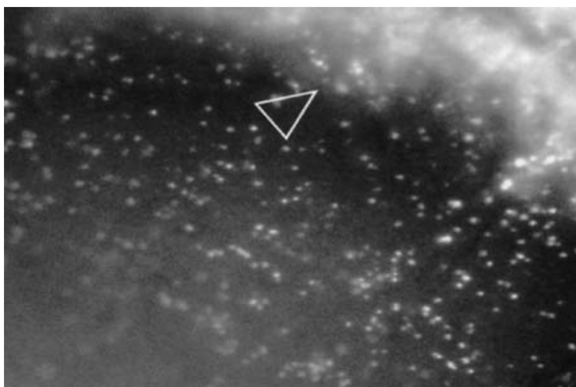

Figure 3 The corneal-limbal area in an eye that had received an injection of mock fluorescent antigen (Cascade-blue Dextran, 70kD) in the anterior chamber 24 hours earlier. Note that many evenly distributed cells in the limbal area and peripheral cornea have taken up fluorescent antigen. Note the very high zone of fluorescence in the area of the irido-corneal angle and sub-conjunctival tissue.

(iris, ciliary body, choroid) of the mouse, rat, and human eye (Figs. 4, 5, and 6). In light of the significant differences in function of macrophages and DCs (vide supra) we shall discuss these separately in the context of the uveal tract.

Macrophages in the Iris, Ciliary Body, and Choroid

Conventional histological and ultrastructural studies revealed macrophages in the human iris stroma to early microscopists. In particular, their propensity to phagocytose melanin shed from iris pigment epithelium throughout life make them a characteristic feature of histological preparations of human eyes where some were thought to represent a subpopulation of “Clump cells” (61). More recently, studies of normal rat and mouse iris, ciliary body, and choroid revealed rich networks (~600–800 cells/mm^2) of RTM (58,62–65) (Figs. 4A,5A,6A). In the rat these cells are CD68$^+$ (ED1$^+$) CD163$^+$ (ED2$^+$) and CD 169$^+$ (ED3$^+$) and in the mouse are F4/80$^+$, CD 169$^+$ (SER4)$^+$, CD11b$^+$58. The success of these studies was due in part to the combination of a wholemount approach to immunostaining (66). The layered structure of the eye particularly lends itself to this approach, which is used extensively in dermatological retinal neurobiological research. The RTMs of the uveal tract display a largely perivascular distribution, suggesting a guardian role at the blood–tissue interface (Fig. 6A). In light of the role of macrophages in mediating innate immune responses by detecting exogenous microbial stimuli via TLRs, mannose receptors, and CD14 (vide supra), this location close to the fenestrated vascular beds of the ciliary body and choroid (but not the iris) would strategically place them as a first line of detection to blood-borne pathogen invasion within the eye.

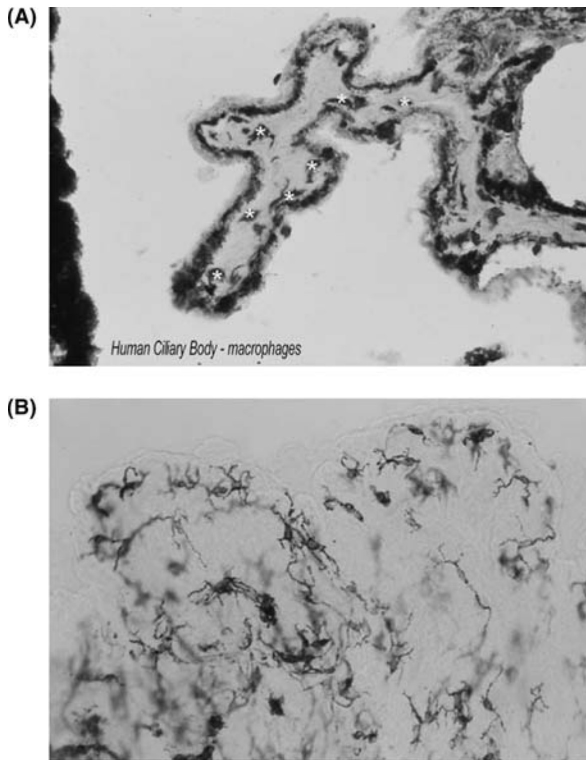

Figure 4 (A) Immunoperoxidase staining of frozen sections of human ciliary processes with an anti-macrophage marker revealing numerous macrophages (*) in the stroma. (B) Rat ciliary process wholemount stained with anti-MHC class II mAb revealing the high density of dendriform stained cells (putative DCs), whose ramifications are finer than those in the iris.

Recent data from our laboratory expands our earlier functional analysis of resident macrophages within the iris (59) and provides insight into the roles of macrophages in primary and secondary immune responses. Several groups have shown that activated macrophages associated with body cavities or mucosal surfaces secrete a range of soluble mediators that inhibit T-cell proliferation (67–69). Using in vitro assays of freshly isolated iris macrophages we noted that they exhibited a functional phenotype that lacked lymphocytostatic properties, but possessed the ability to ingest, process, and effectively present soluble Ag to Ag-specific T cells (70). This functional data suggests that iris macrophages, unlike mucosal or "body cavity" macrophages, do not produce nitric oxide in response to T cell-derived signals, such as IFN-γ. This may be due to their exposure to high concentrations of TGF-β and/or calcitonin gene related peptide (CGRP).

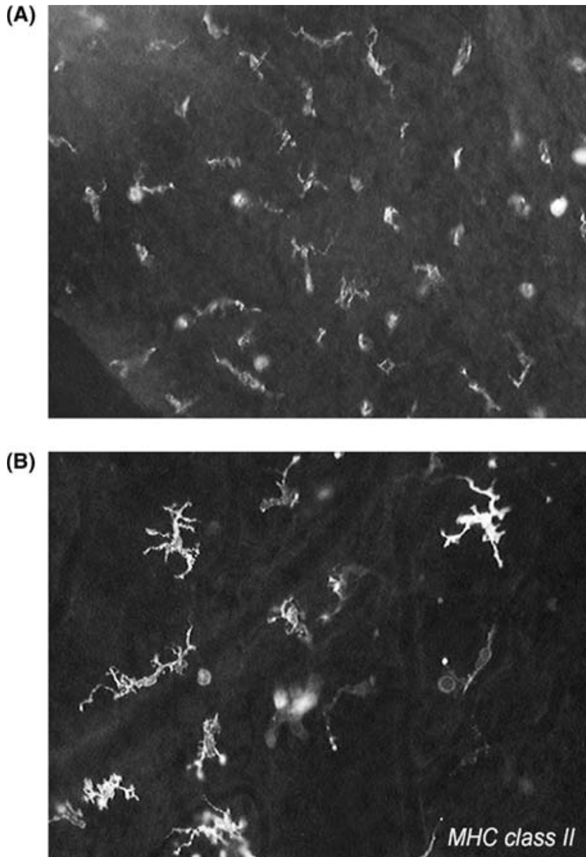

Figure 5 (A) Mouse iris stained with anti-CD169 mAb revealing the network of macrophages. (B) Mouse iris stained with anti-MHC class II mAb to reveal the DC network.

DC in the Uveal Tract of the Eye

Many early studies, performed on conventionally sectioned ocular tissue, either failed to reveal any MHC class II$^+$ cells or revealed only occasional, scattered cells in the normal uveal tract (see review, Ref. 71). In the early and mid-1990s a number of groups, including our own, discovered a contiguous network of MHC class II$^+$ DCs in the iris, ciliary, and choroid of mouse, rat, and human eyes (58,60, 62–65,72). DCs in the uveal tract (Figs. 4B, 5B, and 6B) display a variety of forms from pleomorphic to the characteristic highly dendriform morphology with multiple, often branched, cytoplasmic processes and indented nucleus, i.e., akin to their "cousins" in other tissue sites such as the epidermal Langerhans cells (51) and in the respiratory epithelium (52). They display a regular spaced or contiguous network-like arrangement but do not display as strong a predilection for the

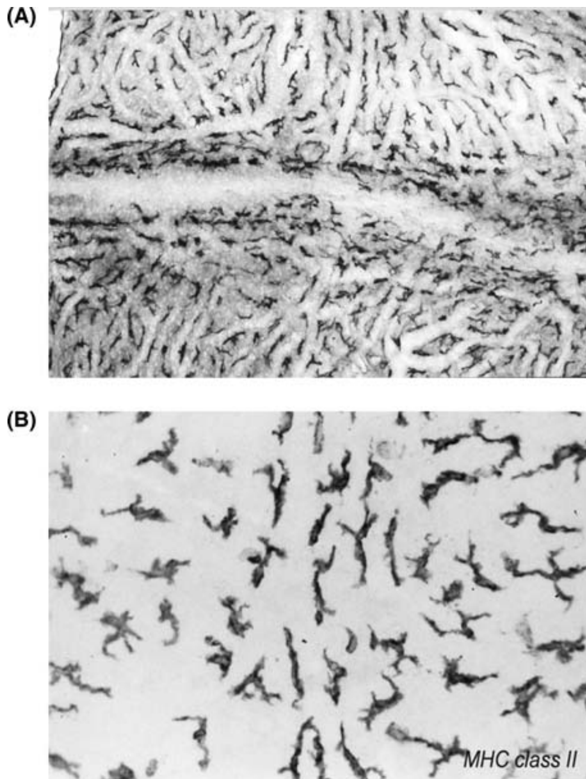

Figure 6 (A) Rat choroid double stained (immunoperoxidase) with anti-CD169 (sialoadhesin, ED3) and anti-CD163 (scavenger receptor B, ED2) mAbs revealing the very high density network of perivascular macrophages which are all double positive. (B) Mouse choroid stained with anti-MHC class II mAb to reveal the DC network. Note the cells are not as strongly orientated along vessels as the macrophages.

perivascular environs as RTMs (Figs. 6A and 6B). The density of the DC network in the mouse and rat iris and choroid (400–600 cells/mm^2) is similar to other well recognized DC populations (e.g., skin 700–800/mm^2, tracheal epithelium 670–880/mm^2, oral mucosa 160–890/mm^2). Double color immunohistochemical studies revealed a lack of macrophage phenotypic markers on the majority of these cells in the rat and mouse eye (58,60), although a small subpopulation of RTMs appear to be MHC classII^{LOW}.

Immunoelectron microscopic and confocal studies have revealed that DCs in the ciliary processes (Fig. 4B) are intraepithelial on the vascular or stromal aspect of the tight junctions that form the blood-aqueous barrier (58). In the choroid, DCs lie directly adjacent to the basal aspect of the retinal pigment epithelium (64). Thus they are strategically situated at the crucial interface between the

choroid and retina and it is tempting to postulate that they may sample Ags either from the intraocular compartment via the basal aspect of the retinal pigment epithelium which forms part of the blood ocular barrier, or blood-borne Ags arriving via the adjacent fenestrated vascular beds. Studies in mice have shown that only a few of the DC in the mouse are $CD80^+$, $CD86^+$, and $\beta2$ $integrin^+$, and even then they are only weakly positive (60), supporting the suggestion that ocular DCs are in the "immature" stage of their life cycle in which their primary role is Ag capture. Despite this postulated role, recent studies in our laboratory have experienced difficulties in detecting any obvious evidence of fluorescent Ag uptake by DCs following injection into the anterior chamber of the eye (73).

TRABECULAR MESHWORK AND OUTFLOW PATHWAYS

There is an extensive literature on the phagocytic properties of trabecular cells (see review, Ref. 71). In addition, the concept of a population of "wandering" phagocytes or RTMs within the trabecular meshwork (Fig. 7) is well accepted and pre-dates the availability of monoclonal antibodies and immunophenotypic analysis. Traditional ultrastructural studies have suggested these cells, which have all the classical morphological characteristics of mononuclear phagocytes, play a role in aiding trabecular cells in the self-cleansing function of the trabecular meshwork. This aids in preventing cellular and extracellular debris (such as melanin, erythrocytes) from accumulating and causing obstruction of the outflow pathways that may compromise aqueous drainage and thus lead to increased outflow

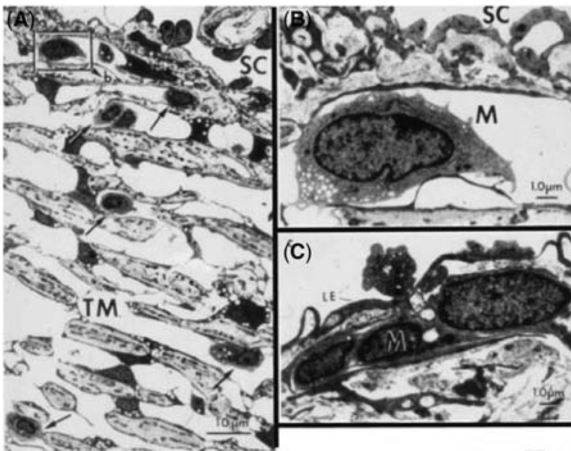

Figure 7 Transmission electron micrographs (**A**, low power; **B**, **C**, high power) of normal human trabecular meshwork (TM) revealing a number of mononuclear cells with characteristics of macrophages (M). Note that these cells are often present in the inter-trabecular spaces (arrows) and occasionally are seen traversing the inner wall of Schlemm's canal (SC) as shown in bottom right panel (**C**).

resistance and the consequential rise in intraocular pressure. Once monoclonal antibodies to various phenotypic cell markers became widely available, it became clear that there were both MHC class II$^+$ dendriform cells (putative DCs) and RTMs within the rat and human trabecular tissues (42,49). To our knowledge there have been no further analysis of the distribution or function of these cell types in the human and non-human conventional aqueous outflow pathways.

Studies in our own (73,74) and another laboratory (75) have shown that following intracameral injection of soluble Ags both free and cell-associated Ags (predominantly within macrophages) can be identified in the trabecular meshwork, iridocorneal angle, and uveoscleral pathways. However, newly recruited CD68$^+$ monocyte/macrophages are also a feature of this model, which involves intraocular injections (a form of injury) and is thus invariably accompanied by a slight degree of inflammation.

Low to moderate densities of MHC class II$^+$ dendriform cells (putative DCs) have been identified in the rat (42) around episcleral vessels and collector channels that serve to drain aqueous humour into the venous blood. DCs at these sites would be ideally located to sample Ags exiting the eye via the conventional aqueous outflow pathway; indeed, recent in vivo video fluorescence microscopy of eyes following intracameral injection of fluorescent-labeled Ags revealed that much of the Ag is deposited or accumulates in the trabecular meshwork and conjunctival/episcleral tissue (Fig. 3). However, phenotypic analysis has shown that the bulk of Ag is trapped or internalized by macrophages (74). It has been known for some time that proteins injected into the AC leak from limbal vessels (76), making it feasible that DCs and macrophages around collector channels and episcleral vessels have access to intracameral Ags. In addition, our data suggest that Ags may pass via the uveoscleral pathways into the loose connective tissue spaces beneath the conjunctiva, a route we postulated several years ago (42). These cells could then migrate, via conjunctival lymphatics, to draining submandibular lymph nodes, thus bypassing the "camero-splenic axis" (route by which Ags in the aqueous humour drain by the venous circulation to the spleen) (2).

Support for the postulated route to draining LNs can be found in the chimera experiments of Egan et al. (77) in which clonal expansion of Ag-specific T cells was noted in the submandibular lymph nodes following intracameral Ag injections. More surprisingly our studies suggest that Ags may also pass to other lymph nodes such as the mesenteric due to Ags entering the blood and thereby becoming accessible to the systemic immune system (78). It must also be considered, however, whether leakage of episcleral and limbal vessels is a consequence of the ocular injury response due to the invasive nature of intracameral injections. We have data that supports this notion.

ARE THERE APCs IN THE RETINA?

While the concept of the blood–retinal barrier (and blood–brain barrier) has been fashioned on the basis of limiting passage of large molecules from the blood

stream into the neural parenchymal tissue (79) it was believed for many years that these barriers naturally extend to blood-borne cells. However, inflammatory processes do occur in the brain and retina and there is evidence that the normal central nervous system (CNS) is subject to regular "patrol" by lymphocytes, most likely of the activated or blast form since resting T cells do not normally enter the CNS parenchyma (80). This is supported by evidence that in autoimmune conditions, such as multiple sclerosis or uveitis, autoreactive T cells do indeed enter the CNS or eye, respectively. A paradigm has emerged that low numbers of lymphocytes, albeit activated, access and "patrol" the CNS parenchyma for potential pathogens (81). If, as evidence to date would indicate, DCs are excluded from the normal neural retina (and brain parenchyma), which cell type within the neural retina acts to present Ag to patrolling activated T cells? If, as outlined earlier, it is accepted that DCs act as the sentinels in the afferent arm of immune responses by sampling Ag in peripheral tissue and regularly migrate to draining lymphoid tissues, one must therefore conclude that this sort of immune surveillance does not occur within the CNS parenchyma and neural retina.

The evolutionary and developmental basis of immune responses within the CNS has recently been reviewed (82). It is convincingly argued that the lack of DCs in the CNS parenchyma (including neural retina) is due to the late evolution of both the adaptive immune system and meninges. The meninges in mammals contain rich populations of macrophages and DCs (83). While primitive meninges are present in cartilaginous fish, the three distinct layers (pia mater, arachnoid, and dura mater) as seen in mammals become identifiable in amphibians and appear to have co-evolved alongside the adaptive immune system. As further support for this co-evolutionary argument, Lowenstein (82) points out that the meninges appear in fetal development around the same time as key elements of the adaptive immune system. It is appealing to consider that similar arguments could be made for the neural retina.

Candidate APCs in the retina include firstly parenchymal cells such as astrocytes, oligodendrocytes, and endothelium and secondly non-parenchymal haematogenous-derived immune cells including microglia (MG) and perivascular macrophages. The potential role of parenchymal cells as APCs in the context of the CNS has been reviewed elsewhere (84). The following discussion will focus on retinal MG and perivascular as these are considered the most likely APCs.

Retinal Microglia

Recent advances in our understanding of MG cells have been derived largely from experimental studies of these cells both in vivo and in vitro. The retina is comparatively flat, accessible, and easily removed from the posterior eye cup. It therefore lends itself to forms of experimental manipulation and examination not possible with the brain. For example, immunostaining of whole retinal flatmount preparations aid in the display of the regular array of MG (Fig. 8) and in vivo confocal scanning ophthalmoscopy allows examination of infiltration of

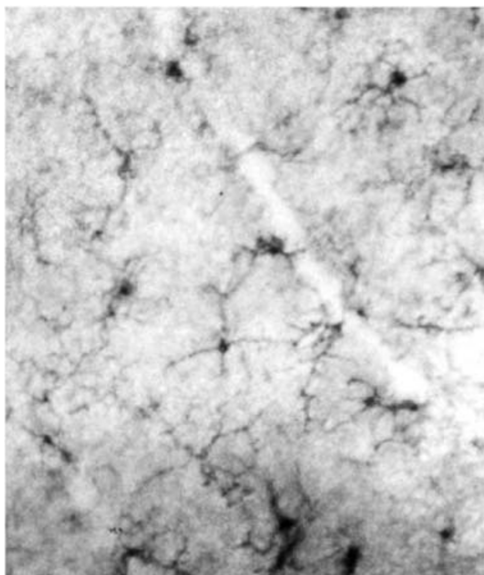

Figure 8 Rat retinal wholemount stained with OX42 (anti-CD11b). Note the highly dendriform nature of the evenly distributed network of cells and the long delicate branched processes.

fluorescent labeled cells in models of autoimmune uveoretinitis (85). Detailed reviews of the history of the discovery, characterisation, origin and nature of MG in the CNS are available elsewhere (86–88).

Microglia are a stable population of highly ramified or dendriform cells of bone marrow origin within the CNS parenchyma that are primarily concerned with innate immune responses and responses to injury (82). They have non-overlapping territories and along with perivascular cells are believed to represent the resident macrophages of the neural parenchyma (89–91). It is estimated that the mouse brain contained 3.5×10^6 MG cells, a figure comparable to the number of macrophages in the liver (92). Within the normal parenchyma of the mouse and rat retina (and brain) MG are CD11b$^+$, CD68^{low} and CD45^{low}. There has been some controversy over whether MG express CD163 [scavenger receptor B, (ED2 in rats)] and CD169 [sialoadhesin (ED3 in rats, SER4 in mouse)], both characteristic of most RTMs in other non-lymphoid organs. The consensus of evidence suggests (42,60,93–95) that retinal MG (and brain MG) are CD163$^+$ but that there may be sub-populations of MG in the perivascular space that have a more classical macrophage phenotype (see 96 for review of full phenotypic profile). These may be thought of as a phenotypically distinct population of macrophages or merely as a subpopulation of MG. The extent of expression of MHC Class II by MG is also controversial (71,96). Studies on retinal MG indicate that they are generally MHC

class II negative at least in mice and most rat strains but in humans retinal parenchymal and perivascular MG appear to be MHC class II^{LOW} (60,62,90,93).

In recent years it has become apparent that DCs, though absent from the CNS parenchyma (including retina), can infiltrate these tissues during inflammation such as multiple sclerosis or experimental autoimmune encephalomyelitis (97,98), and a similar situation appears to exist in the retina during inflammation (99). There is also some evidence that in vitro MG can differentiate into DCs in the prolonged presence of GM-CSF (98); however, whether this can occur in vivo is presently unclear.

Cultured human MG appear to elaborate significant quantities of IL-10 in vitro, supporting an anti-inflammatory rather than a pro-inflammatory role for these cells (100). Indeed, the exclusion of proteins by the blood–brain barrier and blood–retinal barrier are thought in themselves to be partly responsible for limiting MG activation and APC function, which may explain the presence of normal complement of tissue macrophages and DCs in the adjacent meninges and choroid plexus of the brain and uveal tract of the eye, respectively (71,83).

The expression of CD200R and fractalkine receptor (CX_3CR_1) on retinal MG and their ability to produce anti-inflammatory cytokines and mediators such as IL-10, PGF2 and TGF-β would indicate these cells serve to limit CNS inflammation by inhibiting APC function and adaptive immune responses, thus preventing newly recruited T cells differentiating along a Th1 pathway (85,88). Similar data have been obtained in vitro for retinal MG (100).

MG in the resting state have a constitutive role in "cleansing" extracellular fluid in the CNS, for example, by degradation of neurotransmitters, and maintaining a state of "vigilance" by monitoring changes in their extracellular milieu (101). Indeed, pinocytosis is often used as a differential marker for MG. When activated, MG assume a more amoeboid form, upregulate macrophage scavenger receptors, and actively phagocytose cell and tissue debris in a number of situations. These range from normal development, where they phagocytose apoptotic neurons, to a variety of degenerative, traumatic, or inflammatory conditions in the CNS and retina (see review, 86). In the eye, studies of retinal development have shown that monocytes enter the neural retina from the overlying developing vasculature (92), and also from the macrophage populations in the vicinity of the regressing tunica vasculosa lentis (102), the vitreous (103), and the developing ciliary body and peripheral subretinal space (104,105). They then differentiate to form a regularly spaced network of MG in the plexiform layers as far as the outer plexiform layer (86,92,106). In light of the newly emerging transplantation therapies for neurodegenerative conditions of the CNS, such as Parkinson's disease (see review, 107), there has been a renewed interest in the role of MG in the brain as APCs in mediation of rejection. With research on potential retinal transplantation gaining momentum, understanding their role in the retina in mediating rejection events and tissue destruction in the eye is also critical.

The migratory phase of the DC life-cycle is vital to their sentinel function in adaptive immune responses (see reviews, this volume). Therefore, if retinal MG were the equivalent of DCs in the retina, one would naturally predict a short half-life and high turnover. However, quantitative analysis of the normal rate of turnover of retinal MG obtained using radiation chimera models indicates very low turnover (months) (94) similar to CNS MG (108). Thus on the criteria of turnover MG do not appear to have a life cycle akin to DCs.

In conclusion, on the basis of immunophenotypic characteristics, Ag-presenting function, extremely low turnover rates, their phagocytic capacity, and their response to cytokines such as TNF, the most accepted view currently is that MG represent specialized RTMs of the CNS parenchyma and not DCs. In their role as the RTMs of this specialized microenvironment they are crucial in mediating innate immune responses in the CNS while suppressing IFN-γ-mediated nitric oxide production (85) that may be injurious during repair responses in the neural microenvironment. Linking these functional roles with the restricted normal distribution of MG within the retina (e.g., their absence in layers more scleral than the outer limiting membrane) is an interesting challenge. It could simply be the case that the retinal pigment epithelium performs the crucial phagocytic role in the outer retina, thus avoiding the need for MG in the photoreceptor layer.

OTHER MACROPHAGE POPULATIONS IN THE EYE

Vitreous Macrophages or "Hyalocytes"

A little studied population of CD11b$^+$ CD68$^+$ CD163$^+$ RTMs is situated between the inner retinal surface and the vitreous "membrane" (Fig. 9). These cells, sometimes referred to as hyalocytes (109), are considered scavengers of this tissue interface and probably arise from the population of macrophages that phagocytose the hyaloid vessels and tunica vasculosa lentis during development (102). It is worth noting that they may act as a source of contamination in 'retinal preparations' (especially in flow cytometry) if all remnants of the vitreous are not carefully removed. In GM-CSF transgenic mice there are notable lens abnormalities that coincide with large numbers of these macrophages/hyalocytes (110) in the vitreous and perilenticular space. More recently, the role of macrophages in lens development has been highlighted by Hose et al. (103) in rats in which a spontaneous mutation, known as Nuc 1, thought to affect programmed cell death, leads to lens abnormalities.

CONCLUSIONS

Understanding the role of macrophages and DCs in ocular immune responses and in homeostatic mechanisms requires not only a consideration of their phenotype, function, and response to exogenous stimuli that threaten the normal physiological function of the eye, but also a clear elucidation of their distribution in the context

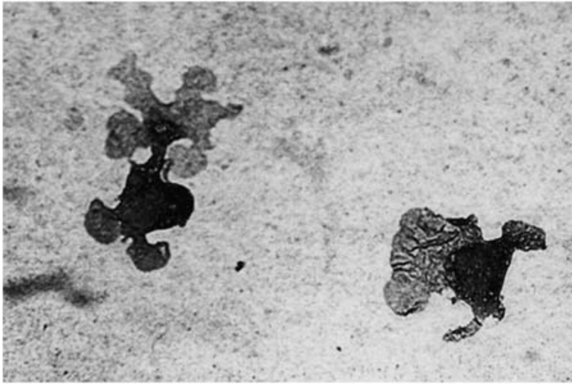

Figure 9 Retinal wholemount stained with anti-CD163 (scavenger receptor B, ED2) mAb and visualized with immunoperoxidase. Note the highly pleomorphic nature of the macrophages (hyalocytes) on the retinal surface. These are not seen if the vitreous membrane is peeled from the retina. Their processes often take the form of veils or blebs. This differs markedly from their close neighbors, the retinal microglia, in the retinal parenchyma, which are CD169- (hence no cells are visible beneath these hyalocytes).

of the complex microanatomical environment of the eye. This review points out that these considerations are especially important in the context of relations of the potential APCs to the blood–ocular barriers in both the posterior and anterior segments, and also in a three-dimensional topographical perspective within a tissue such as the cornea.

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7

Phenotype and Distribution of Antigen-Presenting Cells in the Mouse and Human Eye

Bita Manzouri
Department of Ocular Immunology, Institute of Ophthalmology, University College London, London, U.K.

Santa Jeremy Ono and Masaharu Ohbayashi
Emory Eye Center, School of Medicine, Emory University, Atlanta, Georgia, U.S.A.

INTRODUCTION

Professional antigen-presenting cells (APCs), in their role in acquired immunity, are involved in the initiation and control of the immune response to antigens present at the interface with the environment. They play a critical role in transferring information from the periphery of the organism to lymphoid organs. APCs are a morphologically heterogeneous group and are mainly divided into two systems: the dendritic cells (DCs), which include epidermal Langerhans cells, and the monocyte-macrophage system (1). All of these cell types originate from the pluripotent bone marrow ($CD34^+$) hematopoietic progenitor cells (HPCs). Amongst these, it is the DCs and the Langerhans cell (LCs) that are unique in their ability to prime naïve T cells after the uptake and processing of antigen. DCs have become one of the most studied cells in immunology, having immunotherapeutic potential in malignancy, infections, and autoimmune diseases (2). DC subpopulations are usually found at the interface with the environment

such as the skin, the airways, and the gut, where they capture foreign antigen and then migrate to the draining lymphoid organs to prime naïve T cells (3). In the functionally immature state, i.e., when localized in blood or in non-lymphoid tissue, the capacity of DCs and LCs to take up and process antigen is high, whereas their ability to prime T cells is weak (4). In this phenotype, they show low or absent expression of co-stimulatory and maturation molecules such as CD80 and CD83 as well as low expression of MHC class II molecules (1). During maturation, their ability to take up and process antigen is lost, and there is upregulation of the co-stimulatory molecules with translocation of MHC II to the cell surface (4). Mature DCs are the most potent inducers of primary T-cell responses (5).

CLASSIFICATION OF APCs

The same cell cannot carry out all the multitude of roles attributed to DCs at once, and, consequently, different subsets of DCs that perform different functions have been identified. These DC subsets were initially more readily identifiable in mouse lymphoid organs and peripheral tissues because of the availability of murine tissue and the expression on mouse DCs of markers not present on human DCs. Mature mouse DCs express CD11c along with the co-stimulatory molecules CD80, CD86, and CD40 (6). Furthermore, they have moderate to high surface-levels of MHC class II, the level of expression of which can be further induced on activation. The T-cell markers CD4 and CD8 are also expressed on mouse DCs, allowing for the segregation of the various subtypes (6). Consequently, using these surface markers along with two others (CD11b, CD205), five subtypes have been identified in the lymphoid tissues of uninfected mice (Table 1).

Langerin is a characteristic marker of epidermal LCs, and is found on the Langerhans DCs in mouse lymph nodes. These cells also stain for myeloid markers, including CD11b, and have high levels of expression of MHC class II as well as the co-stimulatory molecules CD40, CD80, and CD86 (6).

Subtyping of DCs is not as well established in humans as in mice due to the relative paucity of DCs being freshly isolated from tissue, with blood being the only readily available source. Human blood DCs have a heterogeneous expression

Table 1 Classification and Localization of the Various Subtypes of Mouse Dendritic Cells

Main site of localization	CD4	CD8	CD205	CD11b	Langerin
Spleen	+	−	−	+	−
Spleen	−	−	−	+	−
Spleen and thymus	−	High	High	−	−
Mesenteric lymph nodes	−	−	+	+	−
Skin draining lymph nodes	−	Low	High	+	+

Source: From Ref. 6.

of a range of markers, but this may be a reflection of the differences in the maturation or activation states of DCs rather than separate subtypes. At least two distinct DC precursor cells have been identified: the myeloid DC (DC1) that carries the myeloid surface antigen marker CD11c, and the lymphoid DC (DC2), which is characterized by a unique surface phenotype of $CD4^+CD3^-IL3R\alpha^{++}HLA$-$DR^+$ (7). Myeloid DCs differentiate from $CD34^+$ HPC or from myeloid precursors, e.g., monocytes (1). Lymphoid DCs derive from $CD4^+CD3^-CD11c^-$ plasmacytoid cells from the blood and tonsils (4). LCs, a specialized type of DC localized to the skin, usually come under the classification of myeloid DCs. However, these cells are increasingly being recognized as a separate DC subtype with distinct markers, including the presence of Birbeck granules and the expression of CD1a and langerin (2,6).

Using the processes of macropinocytosis or endocytosis utilizing a number of cell surface immunoglobulin (Ig) membrane receptors (e.g., $Fc_\gamma RII$ and $Fc_\varepsilon RI$), DCs are able to internalize high molecular weight antigens (4). These antigens are subsequently loaded onto MHC class II molecules after processing. Internalization of low molecular weight haptens occurs via binding to surface glycoproteins leading to their presentation with MHC class I molecules to $CD8^+$ T cells (4).

Following uptake of antigen, the DCs migrate to the regional lymph nodes to present antigen to T cells. Migration and maturation of DCs appear to be linked processes. The migration of DCs to lymphoid organs is tightly regulated by the expression of chemokines in the different anatomical sites as well as the coordinated expression of chemokine receptors on the surface of DCs (4). Interestingly, the chemokine receptor profile expressed on immature DCs (CCR1, CCR2, CCR5, and CCR6) is such that it mainly recognizes chemokines that are released during inflammatory processes (4). During the course of migration, DCs undergo an extensive metamorphosis in their structure and surface phenotype, now appearing as cells with long dendrites. During this maturation, their ability to uptake antigen is lost, with their main purpose now being antigen presentation. Mature DCs express different chemokine receptors (CCR4, CCR7, CXCR4, SLC, and ELC), which allow them to receive signals that will attract them to the regional lymphatics and eventually to the T-cell rich areas of the lymph node (4). Furthermore, there is an upregulation of peptide loaded MHC class I & II and co-stimulatory molecules (CD80, CD86) on the surface of these cells, along with downregulation of Fc receptors. Several factors, such as lipopolysaccharides (LPS), TNF-α and IL-1, are able to induce both of the above processes in vivo (8). This maturation process transforms DCs into particularly potent T-cell stimulatory cells. To achieve this aim, DCs and T cells have to co-localize in the paracortical zone of the lymph nodes, with one single DC being able to prime several hundred naïve T-cells (4). Peptides are presented on the surface of DCs in association with MHC class I or class II molecules to the T-cell receptor complex (TRC). The interaction of the co-stimulatory molecules CD80 and CD86 with their counterparts on T-cells determines whether this stimulation will result in an antigen specific proliferation of T-cells (an immunogenic response for cases of infection with

pathogens) or tolerance (beneficial in cases of harmless environmental proteins or self-proteins) (4,9).

In humans, DC1 cells are responsible for inducing T_H1 cells whereas the DC2 subset of cells induces T_H2 differentiation. Each T_H cell subset has different functions (Table 2). IL-12, secreted by APCs, is the key cytokine that switches T_H cells into T_H1 development (1,4). IFN-γ also promotes T_H1 development, mainly by enhancing IL-12 secretion and partly by stabilizing functional IL-12 receptors on $CD4^+$ T cells (1). On the other hand, the development of T_H2 cells is mainly dependant on IL-4 (4), with the source of production probably being naïve T cells. IL-4 also enhances the maturation of DC1 cells and leads to the apoptosis of immature DC2 cells. IFN-γ from T_H1 cells protects immature DC2s against this IL-4 and IL-10 induced killing and promotes DC2 differentiation. Rissoan et al. (10) have shown that myeloid DCs (DC1) are responsible for driving T cells into T_H1 development, while lymphoid DCs (DC2) direct T cells into T_H2 in an IL-4 independent way.

The critical factor for the polarizing mechanism appears to be the level of IL-12 produced by DC1s, which can be influenced and modulated directly by the pathogen, by micro-environmental factors, and by affecting DC1 maturation at different stages (1). This capacity to influence the type of T-cell response may explain why some antigens induce an allergic response and others do not. The cytokines released during T-cell priming also induce a different chemokine receptor profile on stimulated T-cells. T_H1 cells express CCR1, CCR2, CCR5, CXCR3, and CXCR5, whereas T_H2 cells characteristically express the CCR2, CCR3, CCR4, and CCR5 chemokine receptors (11). The receptor profile expressed may influence the recruitment of these cells to specific types of inflammation and determine what other cells are also recruited to these sites (4).

Atopic individuals show an inherited tendency towards T_H2 responses (1). Various studies have indicated that APCs, and DCs in particular, derived from peripheral blood of atopic patients, have a reduced capability to produce the T_H1 driving factor IL-12, therefore resulting in a bias towards the development of T_H2 cells (12,13). Healthy individuals produce IFN-γ from T_H1 cells upon exposure to allergens, whereas atopic individuals respond with the production of IL-4, IL-5, and IL-13, and reduced production of IFN-γ (9,14). IL-4 and IL-13 are the principal mediators for the production of IgE in B cells, and are therefore key initiators

Table 2 Functions of T_H1 and T_H2 Polarized Cells

T_H1 cells	T_H2 cells
Develop in response to intracellular pathogens	Develop in response to helminths
Produce high levels of IFNγ	Also produce IL-4, IL-5, IL-9, and IL-13
Support development of cytotoxic $CD8^+$ T-cells	
Support production of opsonizing antibodies in B-cells	Support IgG4 and IgE production by B-cells

Abbreviations: IFNγ, interferon γ; Ig, immunoglobulin; IL, interleukin.
Source: From Ref. 9.

of IgE-dependent reactions. IL-5 acts, as an activating cytokine, mainly on eosinophils. These processes account for the high serum levels of IgE and activated eosinophils seen in T_H2 dominant diseases.

IgE RECEPTORS

IgE is known as the main antibody involved in allergic inflammatory processes such as asthma, atopic dermatitis, and allergic rhinitis. Two distinct receptors have been demonstrated for IgE: the high-affinity IgE receptor (FcεRI), and the low-affinity IgE receptor (FcεRII).

In humans, the high-affinity receptor has two forms: the "classical" tetrameric FcεRI ($\alpha\beta\gamma2$), which is constitutively expressed on effector cells of anaphylaxis (mast cells, basophils), and the trimeric form ($\alpha\gamma2$), which is variably expressed on APCs such as monocytes, DCs, and LCs (15). This minimal structure enables APCs to efficiently take up and present antigen in IgE-mediated, delayed-type hypersensitivity reactions that are thought to play an important role in atopic disease. However, the FcεRI in the trimeric form shows much lower density of surface expression and a reduced stability of the receptor protein complexes (16). The α chain of FcεRI is responsible for IgE binding and is a member of the immunoglobulin superfamily (17). The four transmembrane domain β chain increases stability and signaling capacity as well as augmenting the maturation of the α chain and its intracellular trafficking to the cell surface, and the dimer of the signal-transducing γ chain, which is shared by other Fc receptor complexes, carries two immunoreceptor tyrosine-based activation motifs (ITAMs) and is mandatory for the surface expression of the heterotrimeric structure (16,18).

Consequent to expressing the FcεRI $\alpha\gamma2$ form of receptor, APCs show an intracellular accumulation of the α chain, which is presumably caused by the slower maturation and transport process due to the absence of the β chain. Again, consequent to the absence of the β chain, signals transducted by the trimeric receptor are 3–5 times weaker than those mediated by the tetrameric receptor (16). The absence of the β chain indicates that it is not related to the capacity of DCs and LCs to respond to FcεRI mediated activation.

Human FcεRII (known as CD23) is a Ca^{+2} dependant type C-lectin and exists in two forms: CD23a, which is constitutively expressed in B cells and is associated with endocytosis of IgE-coated particles; and CD23b, which is induced in particular by IL-4, is found also on non B-cells such as T cells, monocytes, macrophages, platelets, and eosinophils. The pathophysiological role of this receptor on APCs in relation to allergy remains to be revealed. It may well be involved in antigen uptake and presentation like the FcεRI receptor in atopic patients, but because of its low affinity for monomeric IgE, it has been assumed that this receptor is involved in the binding of IgE-antigen complexes (1).

In atopic individuals, recent studies have shown that FcεRI is the main serum IgE-binding structure on APCs and allows these cells to endocytose IgE-complexed allergen with high efficiency, thereby enabling the threshold dose of allergen required to activate allergen-specific TH cells to be 100–1000x lower

than the dose required for uncomplexed allergen or for nonatopic DCs (9). Hence, antigens are more efficiently taken up, processed, and presented to T cells after targeting to the APC via FcεRI as compared with allergen binding to APC in the conventional manner. After polyvalent ligation, the FcεRI-bound IgE is internalized into acidic proteolytic compartments where it is degraded before delivery to organelles containing MHC class II (16).

The binding of an allergen to the complex of IgE-FcεRI and the associated cross-linking of these receptors on FcεRI-bearing cells leads to the rapid activation and release of inflammatory mediators and the production of a variety of cytokines by APCs. Observations in atopic dermatitis (AD) patients have shown that this resultant cytokine production by the aggregation of surface FcεRI may preferentially induce a T_H2 type of cell activation (17,19).

APCs OF THE ANTERIOR SEGMENT OF THE EYE

Maintaining the integrity of the visual system despite numerous and constantly changing immune challenges is a requirement for vision. The environment of the anterior segment of the eye is unique in that it has DC populations that prevent the development of delayed hypersensitivity responses following antigen invasion into the eye. This evolving concept has been termed "anterior chamber associated immune deviation" (ACAID) and ensures that the eye is able to receive immune protection but that this immune response is devoid of the T cells that mediate delayed hypersensitivity and the antibodies that fix complement (20). The result of this is the sparing of the eye from the potentially blinding influence from ensuing immunogenic inflammation. The concept of ACAID continues to evolve, however, as research sheds further light on the exact nature of the immune cells presents in the anterior chamber of the eye and their cytokine milieu.

The distribution of APCs, especially LCs, appears to be compartmentalized within specific regions of the ocular surface, with the highest concentration being observed in the conjunctiva and peripheral cornea (21). Of the anatomical structures in the eye, it is the cornea that has received a great deal of interest, partly owing to its ability to handle immunity due to its direct contact with the environment and partly due to the information gathered from corneal transplantation research. Previously, it was postulated that the immune behaviour of the cornea was due to the complete absence of MHC class II-bearing DCs from the middle of this organ. Recently, researchers have been able to provide evidence that DC subsets at different precursor and maturation states exist within the corneal tissues, including the central cornea (22). DCs and LCs expressing the cell surface markers $CD80^+$, $CD86^+$, MHC class II^+, and $CD11^+$ have been found to be located in the periphery of the anterior cornea, whereas macrophage-like cells are detectable throughout the whole cornea (23). The immature precursor DCs of the central cornea, although having molecules that clearly identify them as part of the DC lineage of cells (CD45, CD11b, and rarely CD11c), do not express accessory molecules for T-cell stimulation, such as CD40, CD80, CD86, or MHC class II

molecules. Various groups have definitively shown that the cornea in fact possesses its own resident bone marrow derived CD45$^+$ population (22). Hamrah et al. (24) identified CD11c$^+$CD11b$^-$ LCs in the corneal epithelium of the normal cornea that had classical ultrastructural features of epidermal LCs. Both Hamrah et al. (25) and Brissette-Storkus et al. (26) have identified CD45$^+$CD11b$^+$CD11c$^-$ monocytic cells in the corneal stroma. Since these immature precursor subsets of DCs lack MHC II expression, they have been overlooked for a long time, resulting in the previously false conclusion that there were no resident DC populations in the central cornea.

Resident MHC class II-expressing DCs do exist in other tissues of the eye and confer upon these tissues the capacity to capture antigen and deliver it to secondary lymphoid organs. DCs are resident in the ciliary body stroma and associated with the iris pigment epithelium (27). The rat choroid, like the anterior uveal tract, also contains a rich population of DCs. Immunoelectron microscope studies have shown that these DCs have fine processes directly adjacent to the basal aspect of the retinal pigment epithelial cells and Bruch's membrane, giving them an ideal position to sample retinal proteins (28). There are, to date, no immunohistochemical studies of DCs in normal human choroids (27).

Ohbayashi et al. (data submitted for publication, 2006) have recently used double color immunohistochemical staining to demonstrate the distribution of DCs in the anterior segment of the A/J mouse eye (Figs. 1 and 2). Unlike humans, mouse DCs, whether of the myeloid or lymphoid lineage, when mature all express the cell surface marker CD11c with the co-stimulatory molecules CD80, CD86, and CD40 and have moderate to high surface levels of MHC class II. Mouse myeloid DCs also express CD11b, whereas mouse lymphoid DCs express CD8α. Using staining for these two cell surface markers as well as staining for the MHC class II molecule marker, Ohbayashi has shown that CD11c$^+$CD11b$^+$

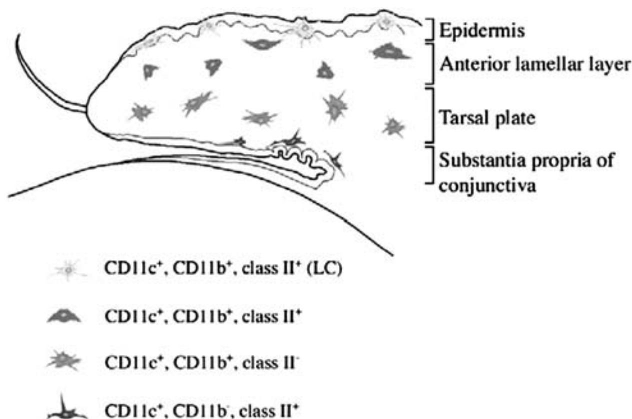

Figure 1 (*See color insert.*) Schematic diagram showing cross section of mouse eyelid with the phenotype of the various cell types as identified by Ohbayashi et al. (data submitted for publication, 2006).

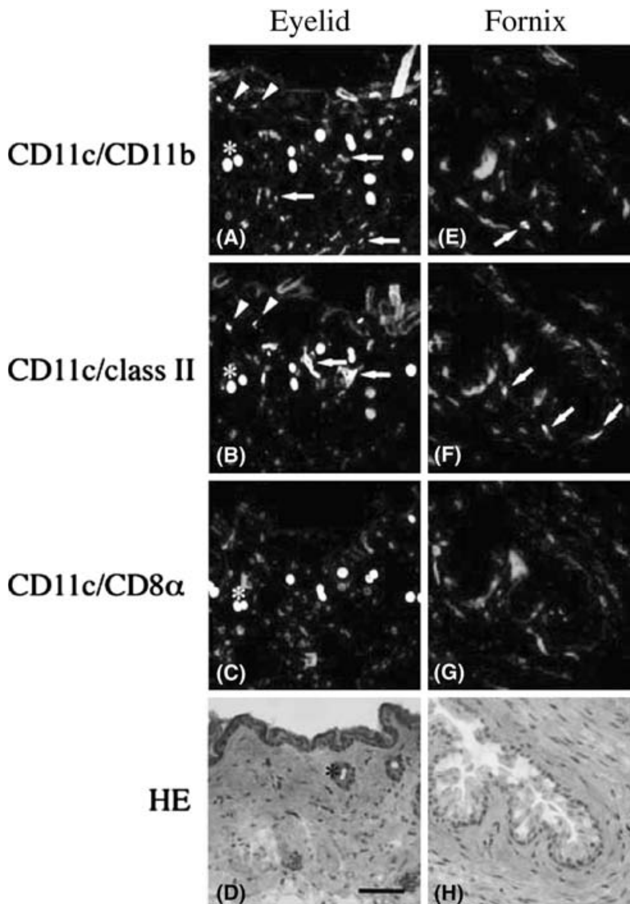

Figure 2 (*See color insert.*) Confocal microscopic analysis of dendritic cells (DCs) in the mouse eyelid section. Serial sections of mouse eyelid tissue were doubly labelled with antibodies against CD11c (red) in combination with (**A** and **E**) anti-CD11b (green); (**B** and **F**) anti-major histocompatibility complex (MHC) class II (green); (**C** and **G**) anti-CD8α; or (**D** and **H**) hematoxylin and eosin (HE) staining. Eyelid regions (**A–D**) and fornix (**E–H**) were analyzed with confocal microscopy. CD11c$^+$CD11b$^+$ LCs in the epidermis (*arrowhead*) and CD11c$^+$CD11b$^+$ anterior lamella (dermis) DCs (*arrow*) are seen at the eyelid area (**A**). Langerhans cells in the epidermis (*arrowhead*) are positive for MHC class II, and anterior lamella DCs in the dermis (*arrow*) are strongly positive for MHC class II (**B**). No CD11c$^+$CD8α^+ DCs are seen both in the epidermis and anterior lamella layers of the eyelid area (**C**). Small number of CD11c$^+$CD11b$^+$ DCs (*arrow*) are seen in the tarsal plate of the posterior lamella of the eyelid (**E**). These CD11c$^+$CD11b$^+$ DCs (*arrow*) do not express MHC class II; CD11c$^+$CD11b$^-$ cells expressing MHC class II were found in the substantia propria of the forniceal conjunctiva (**F**). No CD11c$^+$CD8α^+ are seen in the fornix area of the eyelid (**G**). Autofluorescence was seen by hair follicles (*). The scale bar indicates 50 µm.

cells, markers for myeloid DCs in the mouse, are present on cells that localize to the epidermal cell layer of the eyelid, and these cells go on to express MHC class II, thereby acting like LCs. However, these $CD11c^{+}CD11b^{+}$ cells are also found in the anterior lamellar layer of the eyelid, where they fail to express MHC class II, and continue to express markers of immature myeloid DCs. Staining for markers of lymphoid DCs (CD11c, CD8α) failed to show the presence of these cells in any layer of the eyelid. In the fornix, on the other hand, the odd myeloid DC was found in the tarsal layer of the posterior lamella of the eyelid. Interestingly, $CD11c^{+}$ cells were found in the substantia propria of the conjunctiva that failed to stain for either the myeloid or lymphoid subtypes of DC but did show MHC class II expression (Table 3). The nature of these cells remains to be elucidated. Again, staining for lymphoid DCs failed to show the presence of these cells in any layer of the eyelid.

IgE RECEPTOR CROSS-LINKING AND ACTIVATION OF APCs

The first direct evidence of DC and IgE interaction was via immunohistochemical and immunoelectron microscope demonstrations of IgE bearing LCs in AD patients (29). Subsequently, FcεRI expression in normal human LCs was also demonstrated simultaneously by two groups (30,31). Binding of an allergen to the FcεRI on APCs from an atopic individual who expresses high levels of this receptor leads to receptor cross-linking and subsequent activation of these cells. In nonatopic individuals this is not the case, since the receptor is only expressed at low levels.

Thomas Bieber and his group have shown this to be the mechanism for epidermal LCs in their extensive studies on the mechanisms underlying atopic dermatitis. APC activation results in the release of mediators and cytokines, like MIP-1 and MCP-1, which act to attract more APCs to the site of inflammation (4).

The cross-linking of the FcεRI receptor induces cytokine production, but, for these cellular responses to occur, the activation of the intracellular signaling pathways is required. When the IgE bound receptors are cross-linked by multivalent antigens, tyrosine residues of ITAMs on both the β (in the case of mast cells)

Table 3 Localization of Cells Expressing Various Cell Surface Markers in the Eyelid of the A/J Mouse

	CD11c	CD11b	CD8α	MHC class II
Epidermis	+	+	−	+
Anterior lamellar layer	+	+	−	+
Tarsal plate (with meibomian glands)	+	+	−	−
Substantia propria of conjunctiva	+	−	−	+

Source: Ohbayashi et al., (data submitted for publication, 2006).

and γ chains (on APCs) are transphosphorylated by the src family protein tyrosine kinase (PTK) called lyn. This process is followed by propagation of intracellular signal transduction, recruitment and activation of syk PTK, phosphorylation of protein kinase Cγ1, phophoinositols breakdown, and the elevation of intracellular calcium concentration (16,17). In normal human LCs, FcεRI cross-linking does induce de novo tyrosine phosphorylation of several proteins but the increase in intracellular calcium concentration is not observed. Conversely, in atopes, calcium mobilization via FcεRI cross-linking has been demonstrated in the LCs of these individuals (16,32). This suggests that some steps of the FcεRI-mediated signaling cascade might be upregulated in atopic individuals.

As previously mentioned, DCs play a critical role in the regulation of T_H cell responses via the secretion of various soluble factors and the expression of membrane associated co-stimulatory molecules. Since interaction of allergen with surface bound IgE-FcεRI complex results in the release of inflammatory mediators and upregulates the production of various cytokines, it is conceivable to assume that FcεRI could be a key molecule which connects IgE-mediated allergic reaction and the preferential induction of T_H2 type T-cell activation, as observed in AD patients (17).

The cross-linking of IgE bound to FcεRI on DCs in peripheral blood results in a different response to that seen in LCs in the skin. In blood DCs, this aggregation of IgE-FcεRI results in receptor internalization, antigen proteolysis and transport to the MHC class II compartment-like organelle where peptide loading of the MHC class II occurs. This enables the DCs to present the antigen bound to MHC class II at the secondary lymphoid tissues after their migration through the blood (33).

Recent data have also suggested a role of FcεRI on the differentiation of APCs mediated by factors involved in anti-inflammatory pathways and known to promote a tolerogenic state. The production of the tolerogenic cytokine IL-10 has been induced by the engagement of FcεRI on human monocytes from atopic donors at the beginning of the IL-4/GM-CSF driven culture process, and this prevented the differentiation of the DCs (34). This resulted in the production of macrophage-like cells that were poor stimulators of T cells. This area needs further study to characterize the precise role of FcεRI in the modulation of DCs and the clinical consequences attached to this finding.

CONCLUSION

APCs, in particular DCs and LCS, and their role in the uptake, modification, and presentation of antigen to T cells, have offered new insights into the regulation and control of the allergic response in various body tissues, especially the skin and the eye. The regulation of FcεRI expression on APCs and the role of this IgE receptor in the initiation and control of the immune response as well as the profile of the chemokines produced as a result of allergen exposure have become better defined. It may be possible to generate APCs that express the FcεRI receptor to

induce allergen specific tolerance in patients with uncontrolled allergic responses to common environmental antigens. Other novel therapeutic strategies, some of which have already been explored, include the use of recombinant and synthetic elements of human IgE as competitive inhibitors of the IgE-FcεRI interaction (35). Inhibition of the steps of the signal transduction pathway to control the allergic response may be another approach. However, much remains to be elucidated, for example, how the immune response can be switched from a T_H2 to a T_H1 type in atopic individuals, in order to help develop safe, effective treatments with minimal side effects to combat a range of allergic conditions.

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8

Eye-Associated Lymphoid Tissue in Dry Eye Syndrome

Fiedrich Paulsen and Kristin Jäger
Department of Anatomy and Cell Biology, Martin-Luther-University of Halle-Wittenberg, Halle, Germany

Saadettin Sel
Department of Ophthalmology, Martin-Luther-University of Halle-Wittenberg, Halle, Germany

Philipp Steven
Department of Ophthalmology, UK-SH, Campus Lübeck, Lübeck, Germany

SPECIFIC DEFENSE MECHANISMS IN THE CONJUNCTIVA AND NASOLACRIMAL DUCTS

The epithelia of the ocular surface, the corneal and conjunctival epithelia, as well as the epithelium of the efferent tear ducts, together with the meibomian glands and main and accessory lacrimal glands and lids, comprise a physiological system that was recently summarized under the term lacrimal-ocular surface system (LOS) (1). The LOS is organized to maintain the clarity of the cornea—a homeostatic set-point. Like the systems that represent epithelial interfaces between the internal and external environments, i.e. the gastrointestinal, integumentary, and respiratory systems, the LOS collaborates with the innate and adaptive immune system to respond to microbial invasion. One venue of this collaboration is comprised of the lacrimal glands, conjunctiva, and efferent tear ducts, which are

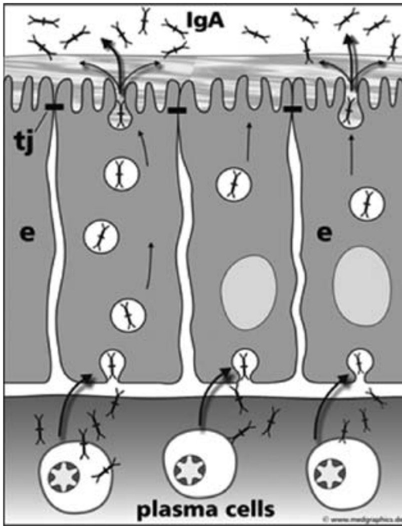

Figure 1 Secretion of IgA by subepithelially located plasma cells, active transepithelial transport of IgA dimers through epithelial cells (e) by binding of the dimers to so-called secretory component and, finally, secretion of the dimers into the nascent tear fluid. Paracellular transport is hindered by tight junctions (tj) between epithelia.

populated by IgA-producing plasma cells and whose epithelia actively transport secretory IgA into the nascent tear fluid (Fig. 1) (2).

Specific secretory immunity depends on sophisticated co-operation between the mucosal B-cell system and an epithelial glycoprotein called the secretory component (3). Initial stimulation of Ig-producing B cells is believed to take place mainly in organized mucosa-associated lymphoid tissue (MALT) (4). It has become evident that MALT is characterized by considerable regionalization or compartmentalization, perhaps being determined by different cellular expression profiles of adhesion molecules and/or the local antigenic repertoire. Antigenic stimulation of B cells results in the generation of predominantly IgA-synthesizing blasts that leave the mucosa via efferent lymphatics, pass through the associated lymph nodes into the thoracic duct, and enter the circulation. The cells then return selectively to the lamina propria as plasma cells or memory B cells (5) by means of homing mechanisms (Fig. 2).

Organized lymphoid tissue in the conjunctiva (conjunctiva-associated lymphoid tissue, or CALT) and efferent tear duct system (tear duct-associated lymphoid tissue or TALT) (6–15) has recently been termed eye-associated lymphoid tissue (EALT) (16). However, aggregated follicles that fulfill the criteria for EALT occur in only just under one-third of conjunctiva and nasolacrimal ducts from unselected cadavers with no known history of disease involving the eye, efferent tear ducts, or nose (7,10,14). In most subepithelial cases, only lymphocytes and other defense cells are amply present inside of conjunctiva and efferent

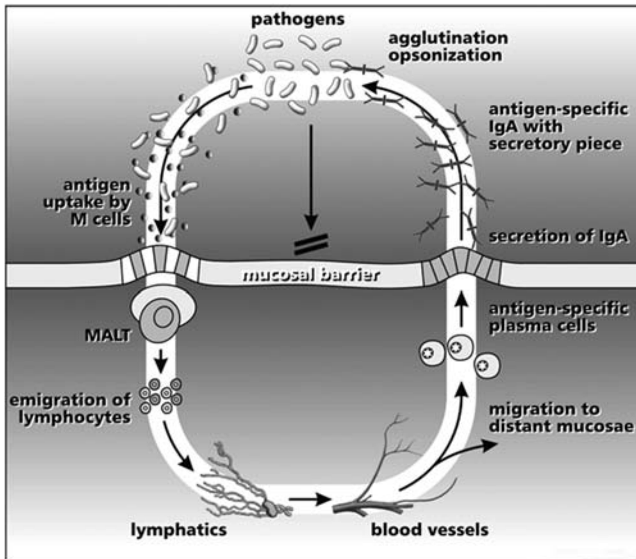

Figure 2 Function of mucosa-associated lymphoid tissue (MALT) (for details see text).

tear ducts not forming aggregated follicles. It is presently still unclear whether special types of bacteria, viruses, allergic reactions, or other factors such as some type of immune deviation are responsible for the development of EALT in humans. However, when EALT is present it may provide the basis for development of primary low-grade B-cell lymphoma of the MALT type.

EYE-ASSOCIATED LYMPHOID TISSUE AS AN ENTRANCE SITE FOR IMMUNOLOGICAL EVENTS

Some organs of the human body (anterior eye chamber, brain, placenta, testicle) are characterized by a special immunological state of reduced activation of the specific and nonspecific immune systems. This condition of local immune suppression, termed the immune privilege, is expressed in delayed or totally suppressed rejection of allogenic transplantations in these organs (17,18); this is illustrated by maintenance of the immunophenotypically immature placenta in the maternal organism as well as in survival of corneal transplants and the immunological acceptance of intraocular lenses. The biological functions of the immune privilege are evident: tolerance of a foreign antigen is obviously better in some organs than its rejection, and this can be achieved only at the expense of T-cell–mediated cytolysis of local cells. Such cell loss is not replaceable in poorly regenerative, postmitotic, or highly differentiated tissues. Therefore, some viruses survive in the central nervous system, as their elimination by T effector cells would certainly lead to neural cell death with severe neurological deficits or even individual

death. A similar situation applies in the anterior eye chamber (19) and testicles. Such immune suppression is not necessary in regenerative organs like the liver or skin, since all cells needed for the process are capable of proliferation and redifferentiation.

The mechanisms that maintain the immune privilege are not uniform in different organs and are not understood in detail. Besides the classical concept of mechanical tissue barriers (i.e., the blood–brain, blood–testis and blood–retina barriers), we must consider the expression of so-called death ligands (CD95, TRAIL, TNF), that induce apoptosis of potentially dangerous T cells as well as a special form of antigen presentation that produces immune tolerance. Such *immune deviation* was first described in the anterior eye chamber (20). Injection of foreign antigen into the chamber does not lead—as at other locations of the body—to a local T-cell reaction (type IV immune reaction), but rather produces systemic tolerance of the inoculated antigen. The antigens are thus not attacked in the anterior eye chamber, in turn protecting the sensitive visual system against inflammatory damage. Thus, the immune privilege of the anterior eye chamber allows transplantation of allogenic lenses, artificial intraocular lenses, and cornea.

Such tolerance is known to be transferable by means of injection into a second animal of splenocytes from an animal primed by antigen inoculation, demonstrating that antigens from the anterior eye chamber receive a signal developed by regulatory T cells that mediates immune deviation. In contrast to the spleen, the cervical lymph nodes do not play a critical role in the induction of immune deviation, as demonstrated in rats by Yamagami and Dana (21). Nevertheless, the drainage routes of the antigens from the anterior eye chamber, the location of their origin, and the passage of the corresponding antigen-presenting cells all remain unclear. In particular, it is not clear what role is played by the conjunctiva and the nasolacrimal ducts and their associated lymphoid tissues [CALT (6,7,14,22–24) and TALT (10–12,15)] in the immune privilege of the anterior chamber of the eye.

Egan et al. (25) demonstrated in mice that potent immunologic tolerance can be achieved by exposure of antigen (ovalbumin) through the conjunctival mucosa. They identified the submandibular lymph node as the principal lymph node in which antigen-bearing antigen presenting cells are located and in which antigen-specific T-cell clonal expansion occurs following conjunctival application of antigen. Clonal expansion was maintained at an elevated level and the T cells were responsive in vitro during a 10-day period of daily ovalbumin application to the conjunctiva. However, in spite of the continuous antigen application, the number of antigen-specific T cells steadily declined over the 10-day period, and by day 14, the remaining ovalbumin-specific T cells were refractory to secondary challenge with ovalbumin, indicating that they had become anergic in vivo. Egan et al. (25) concluded that the fact that antigen-presenting cells presenting ovalbumin were found only in the submandibular lymph node and not in other lymph nodes, spleen, or nasal-associated lymphoid tissue (NALT) rules out the likelihood that tolerance in this system was due to drainage of antigen through the efferent tear ducts and association with NALT or gastrointestinal-associated lymphoid tissue (GALT).

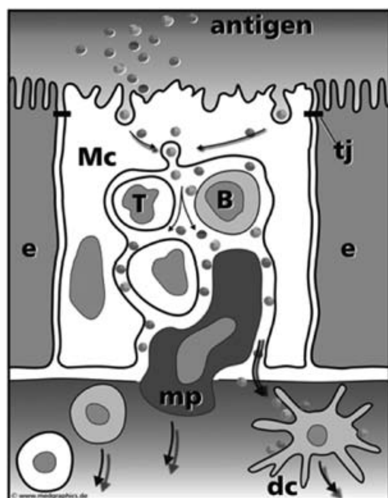

Figure 3 Antigen uptake by an M cell (Mc) (for details see text). *Abbreviations*: e, epithelial cell; T, T lymphocyte; B, B lymphocyte; mp, macrophage; dc, dendritic cell; tj, tight junction.

However, one important point is lacking in the suggestions of Egan et al. (25). It has not yet been taken into consideration that antigens that are drained by the tear fluid itself and not injected intraconjunctivally would be able to induce immune deviation via CALT and/or TALT. With regard to protection of the cornea against inflammatory destruction, this would be plausible and analogous to the process in the nervous system and the anterior eye chamber (20). It is as yet not known how antigen is translocated across the epithelium of conjunctiva and the nasolacrimal duct. M cells, highly specialized epithelial cells that facilitate uptake and transcytosis of macromolecules and microorganisms (Fig. 3), are present in the dome-associated epithelium of peyer's patches. Following transcytosis, antigens to cells of the immune system are released in lymphoid aggregates beneath the epithelium, where antigen processing and presentation and stimulation of specific B and T lymphocytes are achieved (26,27). Whereas epithelial cells of the conjunctiva, which show morphological features of M-cells have been demonstrated in several animal species, no such cells could be demonstrated in humans. The mechanisms of conjunctival antigen uptake and transport remain unclear.

According to a definition by Isaacson (5) for MALT of the gut wall (i.e., Peyer's patches), MALT comprises four components (Fig. 4): (*i*) organized mucosa-associated lymphoid tissue, (*ii*) a lamina propria, (*iii*) intraepithelial lymphocytes, and (*iv*) an associated lymph node. Circulation of the lymphoid cells in these four components enables their homing to their original and other mucosal sites, where they exert the effector function. Such a response may be dominated by sIgA release and may include cytotoxic T-lymphocyte action (27). In this regard, the submandibular lymph node found by Egan et al. (25) could be the "associated lymph node" of CALT and TALT but not of NALT.

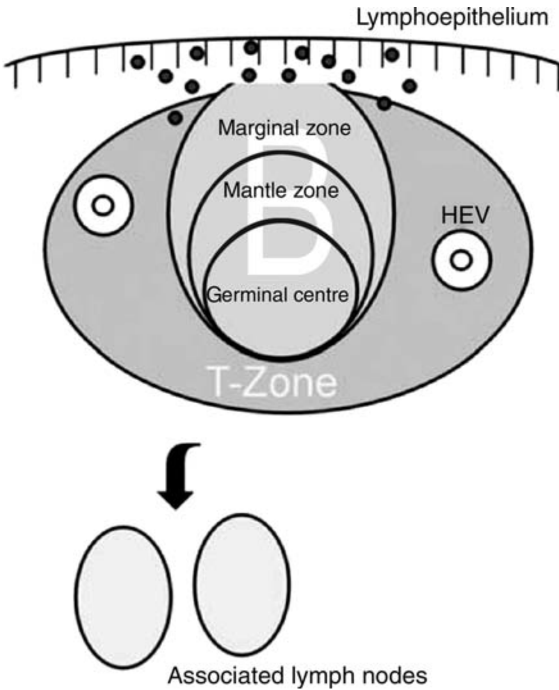

Figure 4 Organization and components of MALT. *Abbreviations*: HEV, high endothelial venules; MALT, mucosa-associated lymphoid tissue.

Activation of T lymphocytes has been observed in dry eye, leading to frequent occurrence of abnormal (pathological) apoptosis in terminally differentiated acinar epithelial cells of the lacrimal gland (28). Tears, now secreted to the ocular surface, will contain pro-inflammatory cytokines and will inflame the tissues of the ocular surface. Abnormal apoptosis has also been detected within the epithelial cells and lymphocytes of the ocular surface (28). This ocular surface inflammatory response consists of inflammatory cell infiltration, activation of the ocular surface epithelium, with increased expression of adhesion molecules, inflammatory cytokines, and pro-apoptotic factors, increased concentrations of inflammatory cytokines in the tear fluid, and increased activity of matrix-degrading enzymes in the tear fluid. It has been suggested that the reduction of circulating androgens plays a role in these processes (29,30). Treatment with locally applied cyclosporin A eye drops interferes with interleukin (IL) metabolism, especially that of IL-6, and thus creates a new treatment option that leads to remarkable improvement of the irritation symptoms and ocular surface signs, especially in severe cases of keratoconjunctivitis sicca.

All these findings lead to the conclusion that CALT and TALT may play a role in the pathogenesis of dry eye. One can imagine that misdirected stimulation of EALT can result in a misguided form of immune deviation at the ocular surface.

Within the scope of this event, apoptosis no longer hinders T-cell autoimmunity induction, completing the picture of dry eye.

It should be mentioned, however, that a recently published article has put our understanding of the functional significance of MALT in a different light. Alpan et al. (31) demonstrated that a systemic immune response to orally administered soluble antigens does not depend on the presence of functional MALT of the gastrointestinal tract, but more likely on initiation of immune response by gut-conditioned dendritic cells. This finding suggests that MALT is not necessary to initiate a primary immune response to antigens that have entered the body. However, if present it seems to act in two ways: (*i*) It produces plasma cell precursors which later migrate into the neighboring mucosa, mature to plasma cells, and produce sIgA for mucosal protection. (*ii*) It allows uptake of antigens by specialized epithelial cells and presentation of these antigens to virgin T and B cells to initiate a primary immune response. Thus, MALT could represent a second pathway (a kind of safeguard of the adaptive immune system) for initiation of an immune response to antigens that have been incorporated into the mucus layer and, in the case of CALT or TALT, have entered the ocular surface and are drained with tear fluid.

It can be concluded that the development of EALT is a common feature frequently occurring in symptomatically normal conjunctiva and nasolacrimal ducts. Whether special types of bacteria, viruses, or other factors, e.g., immune deviation, are responsible for the development of EALT in humans requires further investigation in prospective and experimental studies.

ACKNOWLEDGMENTS

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9

Lacrimal Epithelium Mediates Hormonal Influences on Antigen-Presenting Cells and Lymphocyte Cycles in the Ocular Surface System

Austin K. Mircheff, Yanru Wang, and Magdalena Baladud de Saint Jean

Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

Chuanqing Ding and Joel E. Schechter

Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

INTRODUCTION

The lacrimal glands and ocular surface tissues comprise a physiological system that functions to ensure the quality of the image projected onto the retina. It does this by producing the ocular surface fluid film, which establishes a smooth refractive interface for light entering the eye, and by helping to maintain the cornea in a non-inflamed, non-vascularized, optimally hydrated state. In a sense, the ocular surface system creates and maintains its own local *milieu extérieur*.

The operational principles of physiological systems usually can be understood as conforming to the logic of servomechanisms activated by deviations from homeostatic set-points. The servomechanism in the ocular surface system seems straightforward: sensations generated in the cornea and conjunctiva represent error

signals, which elicit autonomic secretomotor output, which activates secretion of mucins, proteins, electrolytes, and water.

The ocular surface system's adaptations to the threat of infection include the capacity to produce mucins, which prevent microbes from adhering to the epithelial surface; microbiocidal products, such as defensins, lactoferrin, lactoperoxidase, and lysozyme; secretory immunoglobulin A (sIgA), which is a complex of dimeric IgA and the secretory component (SC) of the polymeric immunoglobulin receptor (pIgR); and free SC.

A body of work by groups such as Franklin et al. (1–3), Allansmith et al. (4), Chodosh et al. (5), Knop and Knop (6,7), and Paulsen et al. (8) has made it clear that the ocular surface system's immune defense mechanisms are to a great extent embedded in the broader mucosal immune system. The mucosal immune system's overarching strategy is to neutralize toxins, create a barrier to infection, and eliminate pathogens while avoiding inflammatory responses that might destroy the function of the tissues that are being be protected. sIgA functions importantly in this context because it neutralizes toxins, opsonizes pathogens, prevents adherence to mucosal surfaces, and mediates excretion of antigen-containing immune complexes (9) but does not fix complement and, therefore, does not trigger cytotoxic or other inflammatory responses. Moreover, IgA exerts several immunomodulatory actions: it suppresses neutrophil infiltration and activation, it inhibits monocyte TNF-α and IL-6 secretion, and it upregulates expression of IL-1 receptor antagonist (10,11). These actions may be integrated into negative feedback loops, since TNF-α upregulates epithelial cell pIgR expression (12).

The mucosal immune- and fluid-secreting functions of the ocular surface system are integrated. Secretomotor stimulation elicits increased secretion of sIgA and SC as well as increased secretion of fluid, tear-specific proteins, and mucins. Moreover, it is beginning to appear that the immune tissues of the ocular surface system can be organized in several different functional states, and that different states of immune function are associated with different capacities for epithelial fluid and protein secretion. The different states of immune tissue function are associated with characteristic immunoarchitectures, which include: the normal presence of scattered foci and diffusely distributed lymphocytes and plasma cells (13); increased focal infiltration, formation of germinal center-like aggregates, and formation of classical germinal centers in Sjögren's syndrome (14–17) and graft-versus-host disease (18); dispersal of lymphocytes from foci and increased diffuse infiltration in pregnancy and lactation (19); and increased diffuse infiltration in normal aging (20).

The thesis we develop here is that: (*i*) the functional design features that lacrimal secretory epithelial cells employ to perform the mucosal immune system function of secreting sIgA inevitably entail exocytotic secretion of a heavy burden of autoantigens to the underlying tissue space; (*ii*) lacrimal epithelial cells also secrete paracrine mediators that support IgA^+ cell infiltration and function; (*iii*) the lacrimal epithelial mediators serve additionally to enforce tolerance to the autoantigens that are exposed; and (*iv*) systemic hormonal levels influence the expression of the lacrimal immunomodulatory paracrine mediators in ways that favor different states of immune function.

LACRIMAL SECRETORY EPITHELIAL FUNCTIONAL DESIGN FEATURES THAT EXPOSE AUTOANTIGENS

Dimeric IgA (dIgA) is produced by plasma cells that reside in the subepithelial tissue space of the lacrimal glands. According to a current cellular model, summarized in Figure 1, the internal membrane traffic pathway lacrimal epithelial cells use to transfer IgA to the nascent lacrimal gland fluid intersects with the pathways they use to secrete the proteins they have synthesized themselves and also with the pathways they use to deliver proteins to the lysosomes. dIgA is

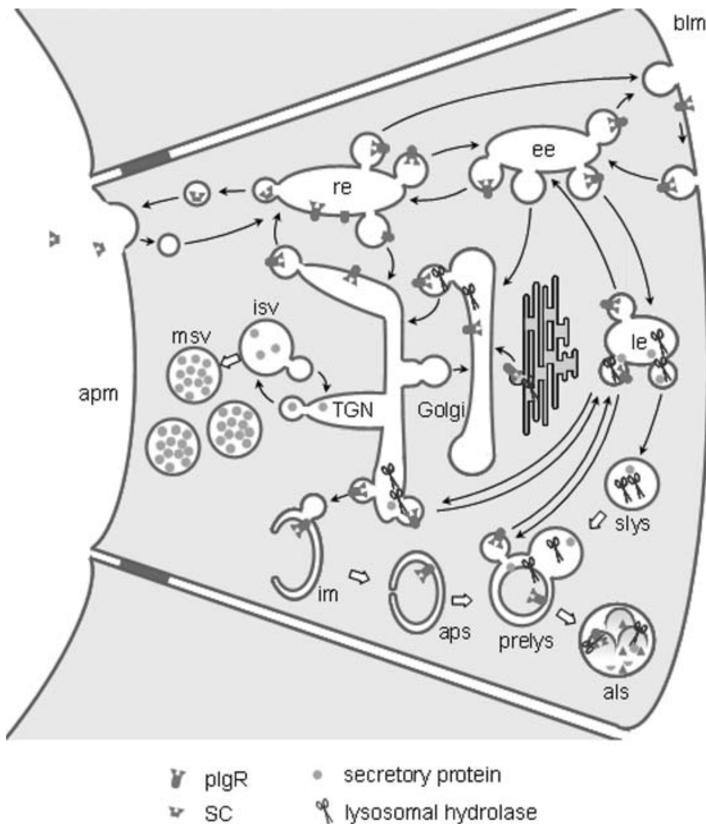

Figure 1 Internal membrane traffic pathways in lacrimal gland acinar cells. In the steady state, pIgR and secretory proteins are replaced by synthesis at the same rate they are lost through secretion and degradation. *Abbreviations*: als, autolysosome; apm, apical plasma membrane; aps, autophagosome; blm, basal-lateral membrane; ee, early endosome; im, isolation membrane; isv, immature secretory vesicle; le, late endosome; msv, mature secretory vesicle; prelys, prelysosome; re, recycling endosome; slys, storage lysosome; TGN, trans-Golgi network.

conducted into lacrimal epithelial cells and through the internal membrane system by pIgR, in the process of transcytosis (21). Like other membrane, lysosomal, and secretory glycoproteins, pIgR is synthesized and modified by addition of simple, mannose-rich carbohydrate groups in the endoplasmic reticulum. The newly synthesized glycoproteins are translocated to the Golgi complex, where their carbohydrate groups are modified, then to the trans-Golgi network (TGN), where they undergo yet more carbohydrate modification, and where they are packaged into transport vesicles that carry them to subsequent compartments.

Transport vesicles carrying newly synthesized pIgR move from the TGN to the common recycling endosome and early endosome, then to the basal-lateral plasma membrane, then back to the endosomes. pIgR that has bound dIgA when it appeared at the basal-lateral membrane is transferred into terminal transcytotic vesicles that move to the apical plasma membrane. At some point it is proteolytically cleaved, releasing SC from the membrane-spanning anchor, and sIgA is released into the lumenal fluid (22). pIgR that fails to bind dIgA may cycle repeatedly between the endosomes, basal-lateral membrane, and TGN before finally moving either to the terminal transcytotic vesicles, where it is cleaved to release free SC, or to the late endosome, prelysosome, and autolysosome, where it is degraded (23).

In the classical merocrine secretory pathway, newly synthesized secretory proteins are transported to immature secretory vesicles, which mature during a process of accretion of contents from an ongoing traffic of TGN-derived transport vesicles balanced by removal of excess membrane, which is thought to return to the TGN. When cells are stimulated with appropriate agonists, some of the mature secretory vesicles fuse with the apical plasma membranes and release their contents into the lumen. Homotypic fusion delivers the contents of additional secretory vesicles. It appears that when cells are stimulated, traffic to immature secretory vesicles may cease, and transport vesicles emerging from the TGN loaded with secretory proteins instead move directly to the apical plasma membrane (24). After the various secretory vesicles have fused with the apical plasma membrane and released their contents, the exhausted secretory vesicle membranes are retrieved and recycled through the Golgi complex and TGN (25).

Some lysosomal proteins move from the TGN to their final destinations by essentially the same pathway as the pIgR that has not bound sIgA, i.e., via the early and recycling endosomes, basal-lateral membrane, and late endosome. Others move directly from the TGN to the late endosome. Transport vesicles that emerge from the late endosome may move to either the storage lysosome or the prelysosome; others may cycle back to the early endosome.

Cytoplasmic proteins, and even intact organelles, enter the lysosomal pathway by way of the autophagosome. This process begins when transport vesicles emerging from the TGN fuse homotypically to form an isolation membrane. The isolation membrane grows through the accretion of more transport vesicles and deforms to enclose an element of cytoplasm, becoming an autophagosome. The autophagosome then fuses with either the storage lysosome or

transport vesicles from the late endosome, forming the prelysosome, which matures to become the autolysosome. The materials that presumably enter the late compartments of the lysosomal pathway via autophagy (26) include autoantigens, such as La/SSB (27) and a number of newly identified dacryoadenitis-related autoantigens (28).

Because the pathway for IgA transcytosis intersects with the lysosomal pathway, lacrimal epithelial cells secrete autoantigens into the underlying tissue space. Secretion of immature lysosomal proteins by vesicles arriving at the plasma membranes from the endosomes is a normal phenomenon. However, lacrimal epithelial cells may secrete an unusually large mass of such proteins because they generate an usually large volume of traffic between their endosomes and basal-lateral membranes in order to secrete sIgA and SC at physiological rates (29,30). Data indicate that La/SSB and other disease-related autoantigens reach earlier compartments of the basal-lateral pathway, i.e., the TGN, early endosome, and common recycling endosome. The flux of autoantigens into these compartments is presumed to be the consequence of communication between the autophagosome, prelysosome, and late endosome.

The challenge that the exocytotically secreted autoantigens pose for the maintenance of self-tolerance may be exacerbated by another functional design feature, associated with the transition between resting and stimulated states. In the resting steady state, ongoing synthesis of secretory proteins is balanced by ongoing degradation in the lysosomes. When cells in an ex vivo model are stimulated with the muscarinic cholinergic agonist, carbachol, traffic to the lysosomes decreases (29,31). Initially, secretory proteins appear to be directed into recruitable secretory transport vesicles, and pIgR may be directed into either the recruitable vesicles or the terminal transcytotic vesicles. Preliminary results suggest that the redirection of traffic from degradative pathways to secretory pathways allows the cell to maintain its pools of secretory products, pIgR, and SC within narrow ranges despite secreting them at 10-fold the resting rate (32). However, when the stimulus is prolonged, the cell's regulated apical secretory pathways become quiescent (23), while traffic to the late endosome remains blocked. It appears that some lysosomal proteins accumulate in immature secretory vesicles, while others accumulate in the TGN and early and common recycling endosomes (28,33). Since immature secretory vesicles communicate with the TGN, products aberrantly accumulating in them, like products accumulating in the endosomes, can be secreted to the surrounding tissue space.

Substantial increases in the rates autoantigens are secreted could challenge peripheral tolerance mechanisms by causing the amount of passively tolerated epitopes presented on MHC Class II molecules to exceed the margin of safety between the relatively low value sufficient to trigger clonal deletion in the thymus and the 100-fold greater value needed to activate T lymphocytes in the peripheral lymphoid tissues (34). The change in intracellular membrane traffic caused by prolonged stimulation also could challenge self-tolerance by exposing epitopes that normally are cryptic. This is suggested because at least some of the lysosomal

proteins that accumulate in the TGN and endosomes are catalytically active proteases. Aberrant processing of autoantigens by these proteases in the endosomes could, conceivably, destroy epitopes that are dominant and expose epitopes that normally are cryptic (35,36). The exposure of previously cryptic autoantigens would challenge active peripheral tolerance mechanisms, because these mechanisms are directed to dominant autoantigen epitopes; cryptic epitopes likely are not subject to active peripheral tolerance. Our thesis is that the active peripheral tolerance mechanisms that operate in the lacrimal glands are integrally related to the glands' mucosal immune function. As a broader context for describing these mechanisms, we will review the organization of mucosal immune system function.

APCs IN INITIATION OF MUCOSAL IMMUNE RESPONSES

Antigens can be taken up across the epithelial linings of various mucosal tissues, and many of these tissues then generate robust sIgA responses. Most of our knowledge about this response has been gained from studies of gut-associated lymphoid tissue in rodents and of human tonsils. The major inductive sites of the mucosal immune system are follicles and organized aggregates of follicles, exemplified by Peyer's patches in the intestine and referred to generically as mucosa-associated lymphoid tissue (MALT). Antigens are often efficiently absorbed by specialized cells in the epithelium overlaying the MALT, the morphologically distinct M cells (37,38). In some cases dendritic cells extend processes between neighboring epithelial cells to sample antigens in the external milieu (39). Despite the well-developed conjunctival lymphoid follicles, however, most of the IgA^+ plasma cells that populate the lacrimal glands appear to be generated in the gut or upper respiratory system, rather than in the eye-associated lymphoid tissues (40,41).

The space subjacent to the M cells contains relatively few dendritic cells but large numbers of immature B cells recently emerged from the follicle. Free antigen and antigen-IgM immune complexes in the extra-follicular region can be taken up by dendritic cells for processing and presentation to the $CD4^+$ T lymphocytes that provide help for activation of naïve B cells. However, most antigen presentation in this region is mediated by activated, MHC Class II^+ B cells that have internalized antigen via surface IgM, which also generates a signal for B-cell survival.

As in the lymph nodes, follicular dendritic cells in the MALT primarily use $Fc\gamma$ receptors to take up antigen-IgM immune complexes. Complement C3 binds to adsorbed immune complexes at the dendritic cell surface and is recognized by CD21 on both the dendritic cells and on B cells, generating additional activation signals. Activated follicular B cells and dendritic cells express CD40, which interacts with CD40L on $CD4^+$ T cells and sustains their activation and production of the T_H2 cytokines, IL-2, IL-4, and IL-10 (42,43). The cumulative signals generated through CD40, cytokine receptors, and surface IgM maintain B-cell activation and stimulate Ig hypermutation (38). Generally, IL-2, IL-4, IL-10, and CD40L together stimulate generation of memory B cells (44). In

contrast, IL-2 and IL-10 in the absence of CD40L stimulate B cells to differentiate as plasmablasts (45,46).

A characteristic phenomenon in the MALT germinal centers is upregulation of B cell J chain expression. In most cases this is accompanied by isotype switching from IgM to IgA, although in the tonsils the switch is preferentially to IgG. The signals responsible for isotype switching to IgA include TGF-β (47–51), IL-10 (52), and vasoactive intestinal polypeptide (VIP) (53,54). It appears that these signals exert a persistent influence on the dendritic cells, since dendritic cells isolated from either Peyer's patch or spleen stimulate antigen-specific T-cell–cognate B-cell monocultures to generate IgA and, secondarily, IgG2 (55).

As B cells mature to the plasmablast stage, they downregulate CXR5 and CCR7, homing receptors that favor retention in the organized tissue, and they upregulate receptors that will favor entry and retention in the effector sites (56). The partially mature plasmablasts exit via efferent lymph vessels, pass through the draining lymph nodes, enter the circulation, and extravasate into the various effector sites of the mucosal immune system: the gastrointestinal tract, respiratory tract, urogenital tract, conjunctiva, and lacrimal drainage system, as well as to various glands: the liver, prostate, salivary glands, lacrimal glands, and, during pregnancy and lactation, the mammary glands.

SIGNALING MILIEUS AT THE EFFECTOR SITES

Specific signals are required to recruit plasmablasts to the effector sites, then stimulate their differentiation to mature plasmacytes and support their ongoing function. Plasmablast differentiation typically requires T cell cytokines. Therefore, the effector sites also generate signals for T-cell recruitment. Evidence that different signals may attract T lymphocytes and IgA^+ plasmablasts to the common effector sites comes in part from studies of small intestine and of mammary gland during pregnancy and lactation. The mucosal address in cell adhesion molecule MAdCAM-1, expressed by endothelial cells, mediates extravasation of T lymphocytes (57,58). In contrast, extravasation of IgA^+ plasma blasts is mediated by CCL25 in salivary glands (59), CCL25, CCL28, and CXCL12 in small intestine, and by CCL28 and CXCL12 in large intestine (60).

Once plasmablasts have extravasated into the effector sites, various signals promote their final maturation to plasma cells and support plasma cell survival and ongoing dIgA secretion. Many of the same signals support expression of pIgR by the overlying epithelium. VIP increases SC secretion by primary cultured lacrimal acinar cells (61). Some signals, e.g., α-MSH, VIP (62–65), and nitric oxide, are released from nerve endings (66). The epithelial cells themselves provide additional critical signals, including IL-6 (67–69), which is an important survival factor for plasma cells, and TGF-β, which supports epithelial function in the mucosal immune system in autocrine fashion, by enhancing epithelial expression of pIgR (70) and IL-6 (71), and in paracrine fashion, by supporting dIgA production (72,73).

It appears that the epithelia at different effector sites may express different spectra of immunomodulatory paracrine mediators in addition to IL-6 and TGF-β. Mammary gland epithelial cells express IL-8 (74) in addition to IL-6 (75,76) and TGF-β1(77). In the liver, parenchymal cells produce IL-5 (78), and duct cells express IL-4 (79). Small intestinal surface epithelial cell lines express IL-8, IL-10, and TNF-α (80,81); they also express receptors for IL-10 (82). Cultured human bronchial epithelial cells express IL-4; during Fas-induced apoptosis they upregulate IL-4 and TGF-β and downregulate IL-6 (83). Conjunctival epithelial cells express IL-1α, IL-8, and TNF-α (84). Human salivary epithelial cells express IL1α (85), IL-1β (86), IL-2 (87), IL-10 (85,87), TNF-α (85,87), and IFN-γ (87). Lacrimal gland ductal cells express TGF-β and prolactin (19), while acinar cells have been reported to express IL-2 (88), and both neuronal- and inducible-nitric oxide synthases, nNOS (89) and iNOS (90).

Systemic hormones support mucosal immune functions at several different effector sites. Ventral prostate and urethral gland epithelial cells express pIgR, and IgA^+ cells populate the underlying tissue spaces, and castration decreases pIgR expression. In the rat, the effect of castration can be reversed by either estradiol or dihydrotestosterone, but neither hormone has a substantial effect on IgA^+ cell content (91). In contrast, testosterone prevents the effects of castration on both pIgR expression and IgA^+ cell number in the mouse prostate and urethral glands (92). To the extent data on the mouse prostate and urethral glands are available, they resemble the extensive body of data on hormonal influences on pIgR expression and IgA^+ cell infiltration in the rodent lacrimal gland that has been published by Sullivan et al. (93,94).

The pattern of systemic hormonal influences on secretory immune functions in the mammary glands differs markedly from the patterns in the lacrimal glands and prostate. Estrogen and progesterone, alone or in combination, have little direct effect on mammary gland epithelial development, pIgR expression, or IgA^+ cell accumulation (95–97). However, they can inhibit conversion of TGF-β from the latent to the active form (98), permitting prolactin to induce epithelial proliferation in some (95), but not all (96,97), species. Prolactin both stimulates accumulation of IgA^+ cells and expression of pIgR by epithelial cells in the intact mammary gland. Notably, these actions of prolactin are inhibited by testosterone, which also inhibits the actions of estrogen and progesterone (95).

Prolactin receptors couple to several signaling pathways, including those initiated by activation of Jak2, Fyn, Tec, SHP-2, Vav, and SOCS (99), and prolactin appears to have important influences on B cells. In an early experiment, an 18–70 kDa fraction of a placental extract increased the numbers of IgA^+ cells and increased IgA secretion in LPS-stimulated spleen cell cultures, and this activity was abrogated by antibodies to prolactin (100). Prolactin stimulated B-cell expansion in bone marrow cell primary cultures (101). Prolactin counteracted the inhibitory effect of TGF-β and enhanced the stimulatory effects of IL-4, IL-5, and IL-6 on mouse B-cell hybridoma proliferation (102). When some, but not all, strains of mice transgenic for an anti-DNA antibody heavy chain antibody are

treated with prolactin for four weeks, they develop a lupus-like syndrome, with elevated anti-DNA titers, increased numbers of anti-DNA B cells, and an overall shift of B cells from the transitional compartment to the follicular and marginal zone compartments (103). Therefore, it appears that interactions between prolactin and TGF-β take different forms to support mucosal immune function in different tissues.

APCs ALSO TRAFFIC TO THE EFFECTOR SITES

Immature dendritic cells also enter the effector sites of the mucosal immune system (104). Large numbers are present in the lamina propria of the intestines (105) and respiratory system (106), and significant numbers also are present in liver (107,108), salivary glands (109,110), lacrimal glands (13,111), and conjunctiva (111,112). In the present context, it may be appropriate to note that they also are present in the cornea (113,114). The immature dendritic cells' constitutive phagocytic activity loads them with apoptotic cell fragments, necrotic cell debris, and soluble autoantigens, as well as foreign antigens (115,117). To some extent, they may then migrate laterally, to the MALT, where, if activated, they would help initiate mucosal immune responses. Primarily, though, they migrate to the lymph nodes and spleen, where, if activated, they would help initiate systemic immune responses (118,119).

Rather than activation, however, the default condition for dendritic cells leaving peripheral tissues is either an immature or partially mature state, in which they express only low levels of MHC Class II, CD40, and B7 and, therefore, are unable to activate naïve T cells (120,124). Such immature dendritic cells are thought to play important roles, both indirectly and directly, in the maintenance of tolerance to the autoantigens they have taken up. It has been suggested that immature dendritic cells regurgitate autoantigens to MHC Class II$^+$,CD8$^-$ dendritic cells that reside in the lymph nodes and spleen. The CD8$^-$ dendritic cells express Fas ligand, and they trigger apoptosis of T lymphocytes that, upon initial activation, are induced to express Fas (125). This mechanism for enforcing tolerance to autoantigens resembles a peripheral version of clonal deletion (126). Furthermore, partially mature dendritic cells express enough MHC Class II to present peptides to T cells, but not enough CD40 or B7 to stimulate naïve T cells to differentiate as memory or effector cells. As a consequence, they cause naïve autoantigen-specific T cells to become anergic (127,131), or to differentiate as IL-10-secreting regulatory cells (132).

The specialized signaling milieus that support plasma cell function and immunoglobulin transcytosis at the effector sites also condition the immature dendritic cells that circulate to these sites. Dendritic cells that have passed through the IL-10–rich milieu of the respiratory mucosa may generate either anergic T cells (133,134) or IL-10–secreting regulatory CD4$^+$ T cells (135,136). The IL-10–secreting cells maintain themselves, as well as bystander cells, in a nonproliferating state by downregulating CD28-mediated signaling (137). They are

referred to as TR1 regulatory cells (138); their regulatory function is thought to maintain normal tolerance to innocuous mucosal antigens and to avert allergic reactions to potentially harmful antigens (106,139,140).

A somewhat different form of tolerance arises when dendritic cells have been conditioned in the TGF-β-rich milieu of the intestinal mucosa (141). Oral administration of antigens generates Peyer's patch T cells that enhance IgA responses and spleen T cells that suppress IgG responses (142). When antigens are fed at low doses, they elicit regulatory lymphocytes, designated T_H3 cells, which secrete high levels of TGF-β (143,144). TGF-β is a decisive signal for generation of TGF-β-secreting T_H3 cells. This phenomenon can be recapitulated ex vivo by priming naïve T cells with irradiated antigen-presenting cells and IL-2 in the presence of TGF-β. The resulting T cells secrete TGF-β upon re-stimulation with anti-CD3, anti-CD28, and IL-2 (145). Two experiments indicate that oral tolerance is a function of the dendritic cells that migrate from the lamina propria to the mesenteric lymph nodes, rather than those in Peyer's patches. First, mesenteric lymph node dendritic cells from µMT mice, which lack B cells and MALT, elicit $CD4^+$ T cells that resemble T_H3 cells in expressing IL-4 and TGF-β, and not expressing IFN-γ (119). Second, it is possible to prevent development of mesenteric lymph nodes but not Peyer's patches in the offspring of pregnant lymphotoxin α –/– mice by treating them with an agonistic anti-lymphotoxin β receptor monoclonal antibody. In contrast, it is not possible to induce oral tolerance in mice that have been prevented from developing both mesenteric lymph nodes and Peyer's patches (146).

Weiner has suggested that high levels of IL-4 and IL-10 converge with TGF-β in the intestinal lamina propria to generate the dendritic cells that direct eneration of T_H3 cells (144). A very large body of evidence for the notion that the local milieu programs antigen-presenting cells to generate T_H3 regulatory cells comes from studies of the anterior chamber-associated immune deviation (ACAID). Treatment of antigen-presenting cells with TGF-β causes them to direct naïve ovalbumin-specific T cells to differentiate as TGF-β-secreting cells (147). This treatment causes several changes in cytokine and co-stimulatory molecule expression in the antigen-presenting cells, including downregulation of CD40 (148) and IL-12 (148,149) and upregulation of IL-10 (149) and thrombospondin-1 (150). Streilein et al. have proposed that thrombospondin-1 then binds to CD36, the scavenging receptor typically expressed at high levels on the surfaces of macrophages and immature dendritic cells, and that the CD36-thrombospondin-1 complex binds active TGF-β in a state that allows it to interact with its own receptor, activating a positive autocrine signaling loop (150).

Regulatory cells that secrete TGF-β have the ability to generate additional TGF-β- and IL-10-secreting regulatory cells. This phenomenon has been called "infectious tolerance." However, Horwitz et al. have observed that generation of T_H3 regulatory cells is prevented by depletion of the small population of peripheral blood $CD4^+$ lymphocytes that express the activation marker, CD25 (151,152).

Peripheral blood $CD4^+$, $CD25^+$ cells have been referred to as "natural regulatory T lymphocytes," or "T_RN" cells. They apparently are generated in the thymus during the early postnatal period, in response to antigens presented by thymic epithelial cells (153–155), which may mimic immature dendritic cells in expressing MHC Class II molecules but not CD40 or B7. The Horwitz group's observations suggest that tolerogenic dendritic cells must necessarily collaborate with pre-existing T_RN cells in order to generate T_H3 cells.

RESPONDING TO DANGER

The default response of the inductive sites of the mucosal immune system is decidedly anti-inflammatory, if not tolerogenic. The default response elicited by dendritic cells entering the lymph nodes and spleen from the effector sites of mucosal immune system is actively tolerogenic. However, several different kinds of signal induce immature dendritic cells to mature with phenotypes that elicit effector responses. While infection of dendritic cells by certain pathogens can downregulate expression of IL-12 (156) or induce production of IL-10 (157,158), most pathogens upregulate IL-12 production (159). LPS can induce dendritic cells to mature with either the so-called DC1 or DC2 phenotypes, which generate T_H1 or T_H2 effector cells. LPS stimulation of bone marrow-derived dendritic cells activates cyclooxygenase-2, and the resulting PGE_2 stimulates production of IL-10 (160), which supports generation of T_H2 effector cells. LPS stimulation of peripheral blood dendritic cells upregulates expression of MHC Class II, B7-1, B7-2, and CD40, and it also stimulates expression of TNF-α, IL-6, IL-8, IL-12 (161), leading to efficient generation of T_H1 effector cells. Stimulation of long-term cultures of spleen-derived dendritic cells with TNF-α and IL-1β induces them to express MHC Class II, B7-2, CD40, and IL-12 (162). Bone marrow cells are activated by necrotic cells, but not by apoptotic cells (163). This may be because necrotic cells release Hsp 70, which stimulates dendritic cell antigen uptake and maturation (164). Interestingly, dendritic cell maturation also is activated by mechanical stress (163) (Fig. 2).

Once a cellular immune response has been initiated, CD40L on effector T lymphocyte also may activate CD40 on immature DCs to stimulate their activation (165,166). DCs that are activated by infection, an inflammatory signaling milieu, or CD40 stimulation are likely to be loaded with autoantigens as well as with foreign antigens. Autoantigen-specific T_RN regulatory cells as well as the regulatory T cells that have been generated under previous, non-inflamed conditions, tend to prevent DC1 and DC2 DCs loaded with auto-antigens from activating naïve autoantigen-specific T cells. However, CD40 signaling in the presence of TNF-α overcomes the tolerogenic influence of IL-10 (165). T_RN cells, which secrete IL-10, lose their capacity to function as regulatory cells in the presence of high concentrations of IL-2. T_H3 regulatory cells appear more robust.

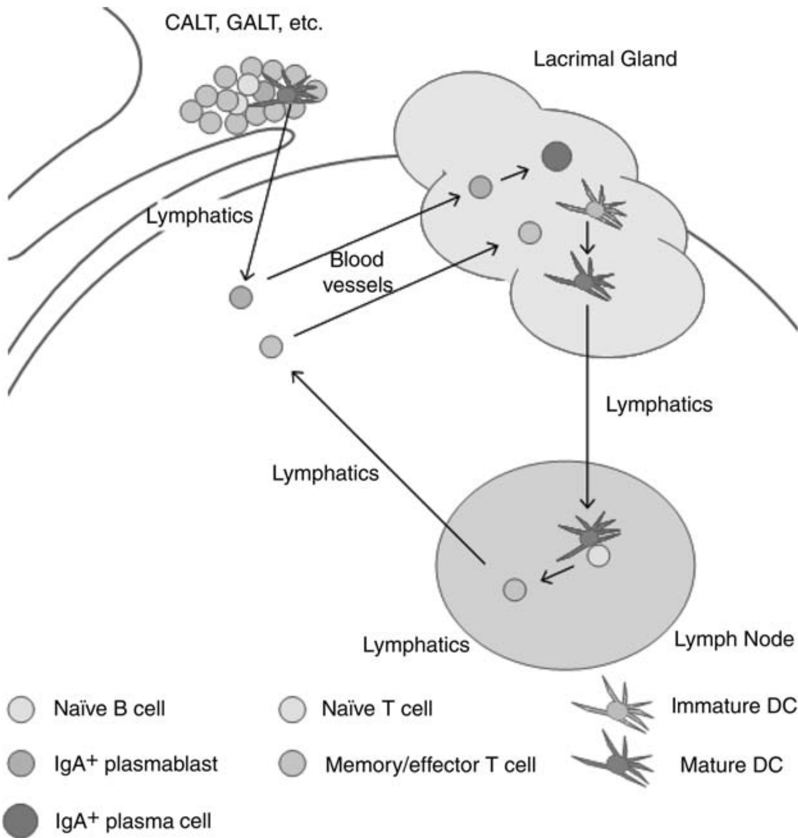

Figure 2 (*See color insert.*) Lymphocyte and dendritic cell (DC) cycles in the ocular surface system. Naïve B cells are activated in the mucosa-associated lymphoid tissues (CALT, GALT, etc.) under the influence of antigens presented by mature dendritic cells (DCs) and activated B cells (not shown). They enter the lymphatics as IgA^+ plasmablasts, then migrate to the lacrimal glands, where they mature as IgA^+ plasma cells. Immature DCs enter the lacrimal glands, become loaded with lacrimal autoantigens, and, under the influence of the local milieu, mature to either an effector-activating or a tolerogenic phenotype. Mature DCs migrate from the lacrimal gland (and conjunctival space) to the lymph nodes, where, depending on their phenotype, they activate naïve T cells to mature with memory, effector, or regulatory phenotypes.

IMMUNOREGULATION IN THE OCULAR SURFACE SYSTEM

Upon arriving in the lacrimal glands, immature DCs normally encounter a milieu that supports plasmacyte maturation, plasma cell survival, and epithelial cell expression of pIgR. This signaling milieu likely stimulates them to mature with a tolerogenic phenotype, and when they subsequently migrate to the lymph nodes and spleen, they should generate regulatory lymphocytes specific for the lacrimal

autoantigens they have acquired. As noted, the ability to generate a milieu that enforces tolerance to autoantigens may be especially important in the lacrimal glands, because their mechanism for secreting IgA entails the exposure of an especially heavy burden of autoantigens.

The same signals that program DCs to mature with activated phenotypes that generate T_H1 or T_H2 responses, instead of T_R1 or T_H3 responses, would threaten to overcome self-tolerance in the lacrimal glands as in any other tissue. The risk may be greatest in the accessory lacrimal glands, which are embedded in the conjunctiva and, therefore, proximate to the likely site of infection and injury and the resulting inflammatory responses.

Endogenous, systemic factors also may adversely influence the lacrimal glands' ability to generate tolerogenic DCs and regulatory T lymphocytes. Prolactin is a paracrine mediator in the local milieu that seems to especially warrant scrutiny. It has been implicated in a number of immunomodulatory roles in addition to support of mammary epithelial pIgR expression and IgA^+ plasma cell accumulation. Treatment with bromocriptine, which suppresses pituitary prolactin secretion, suppresses contact sensitivity, antibody formation, adjuvant arthritis, experimental allergic encephalitis (167) and induction of tumoricidal macrophages (168).

At physiological levels, prolactin has similar effects to IL-4 in stimulating the GM-CSF-dependent maturation of peripheral blood monocytes to DCs that express CD80, CD86, and CD40 and are capable of activating allogeneic T cells (169). These actions result in part from prolactin-induced upregulation of GM-CSF receptors. High concentrations of prolactin induce differentiated DCs to secrete IL-12. It has been proposed that this reflects a positive feedback loop in which prolactin-dependent upregulation of DC CD40 increases responsiveness to T cell CD40L (170). Prolactin stimulates maturation of DCs in cultured fetal thymus explants, and it induces isolated adult thymic DCs to upregulate MHC molecule and CD80 expression, as well as secretion of IL-1, IL-12, and TNF-α (171). Prolactin also induces expression of IL-1α and TNF-α by astrocytes (172). Therefore, it seems possible that systemic hormonal changes that alter local prolactin levels in the lacrimal glands may lead to generation of DCs that elicit T_H1, rather than T_H3, responses.

Elevated, but not excessive, physiological systemic prolactin levels also may influence the outcome of antigen presentation when DCs traffic from the lacrimal glands to the lymph nodes and spleen. Prolactin induces expression of IL-2 receptors (173) and stimulates proliferation (174) in spleen cell cultures from ovariectomized female rats. Administration of estrogen overcomes this effect. In whole blood cell cultures incubated in the presence of ionomycin and phorbol myristate acetate, prolactin increases the number of $CD4^+$ cells that express the T_H1 cytokines TNF-α and IFN-γ, and it increases the number of $CD8^+$ cells that express IL-2 as well as TNF-α and IFN-γ (175). In whole blood cells stimulated in the combined presence of PHA and LPS, physiological levels of prolactin increase secretion of IFN-γ and IL-12, but not IL-10 or TNF-α, while

supraphysiological levels have no effect. In the presence of LPS alone, prolactin increases secretion of IL-10 but not IFN-γ, IL-12, or TNF-α. Prolactin enhances IL-2-dependent synthesis of IL-12 by NK cells at physiological, but not supraphysiological, levels (176). It has biphasic effects on DC-mediated generation of cytotoxic T lymphocytes ex vivo, increasing the response in the physiological concentration range of 12–25 ng/ml and suppressing the response at the supraphysiological concentration of 200 ng/ml (177). In Nb2 T lymphoma cells, prolactin signaling through Jak can lead to activation of Stat1 and upregulation of interferon regulatory actor-1 (IRF-1), stimulation of IL-2 production, and proliferation (178), or to activation of Stat2 and repression of IRF-1 (179).

If DCs conditioned in a prolactin-dominated lacrimal milieu generate effector or memory T lymphocytes in the lymph nodes and spleen, those lymphocytes will likely encounter signals that retain them in the lacrimal gland. TARC (thymus activation regulated chemokine, CCL17) and MDC (macrophage-derived chemokine, CCL22) are expressed constitutively in the lacrimal glands (180). The T lymphocytes that extravasate into the lacrimal glands will interact with recently arrived immature DCs as well as mature DCs that have not yet emigrated to the lymph nodes. The levels of prolactin in the milieu of the lacrimal gland may influence the results of those interactions. Prolactin prevents glucocorticoid-induced apoptosis (181) and it can replace IL-2 in stimulating proliferation of Nb2 lymphoma cells, where its actions include upregulation of pim-1 (182), which is a cytokine-induced protein kinase involved in lymphocyte activation, and the anti-apoptotic factor, Bcl-2 (183). Although prolactin transiently upregulates message levels for the pro-apoptotic gene, *bax*, it does not increase, and in some cases it actually decreases, expression of Bax protein (183). Activation of memory cells and proliferation of effector cells in the lacrimal glands would then likely increase expression of chemokines that further increase recruitment of lymphocytes. The T_H2 cell-derived cytokine, MCP-1 (monocyte chemoattractant protein), increases as disease progresses in MRL/MpJ mice (180), while RANTES, IP-10, and lymphotactin are associated with lymphocyte accumulation in NOD mice (184).

The cytokines released from activated lymphocytes then also may induce or up-regulate cytokine expression by glandular epithelial cells. Inflammatory cytokines increase intestinal epithelial cell expression of MCP-1, GM-CSF, TNF-α, and IL-8 (185,186). In salivary glands of NOD mice focal lymphocytic infiltration is associated with increased epithelial cell expression of IL-1β, IL-2, IFN-γ, and TNF-α, in addition to IL-10 and, in some cases, IL-4 (187). Similarly, the levels of IL-1α, IL-1β, IL-2, IL-6, TNF-α, IFN-γ, and TGF-β are markedly increased in salivary gland epithelial cells from Sjögren's syndrome patients (85–87). IL-1α, IL-6, IL-8, TNF-α, and TGF-β also are elevated in conjunctival epithelial cells from Sjögren's syndrome patients (84). Epithelial cells also may be induced to express C-reactive protein, serum amyloid P component, H-kininogen, and T-kininogen (188,189), mediators that normally are released by mast cells (190). It is possible that inflammatory processes outside the ocular surface system also induce lacrimal epithelial cells to express the acute phase

mediators, since their mRNAs are upregulated in the submandibular and lacrimal glands of rats that have been injected subcutaneously with turpentine oil (191).

If alterations in local or systemic prolactin expression increase the tendency for activation of DCs and initiation of T_H1 effector responses, local expression of TGF-β may exert a countervailing influence. It is intriguing to consider that prolactin and TGF-β act upon each other in the local signaling milieu. On the one hand, prolactin inhibits expression of TGF-β by breast cancer cells (192). On the other hand, TGF-β inhibits expression of prolactin by anterior pituitary cells (193), decidualized endometrial cells (194), and GH3 cells (195). As illustrated in Figure 3, immunopositivities for both prolactin immunoreactivity and total TGF-β increase markedly within ductal epithelial cells during pregnancy (19). Preliminary results suggest that increases in systemic estrogen and progesterone levels may overcome the mutually inhibitory actions of TGF-β and prolactin, since administration of estrogen and progesterone to ovariectomized rabbits appears to elicit the same pattern of changes in prolactin and TGF-β level and expression (JE Schechter and C Ding, unpublished observations).

One can imagine that the levels of locally expressed prolactin and TGF-β, as well as IL-2 produced by epithelial cells and prolactin produced by the pituitary gland, influence the state of immunoregulation in the lacrimal glands. One program of coordinated local signaling changes occurs in pregnancy, which, in rabbits, is associated with a change in immunoarchitecture that is quite distinct from the changes in Sjögren's syndrome, but, nonetheless, accompanied by profound impairments of fluid and electrolyte secretion (196). A different pattern of changes may occur during normal aging, when hormonal support for local

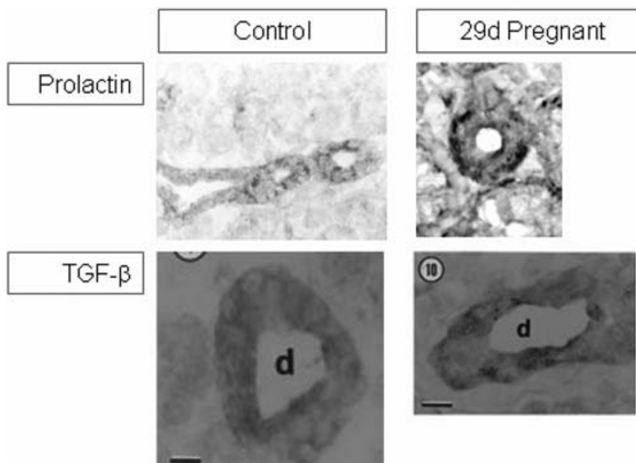

Figure 3 Immunomodulatory paracrine mediators in the lacrimal glands. Ductal epithelial cells express TGF-β and prolactin, and the levels of both increase dramatically during pregnancy. *Source*: From Ref. 19.

prolactin and TGF-β expression decreases but pituitary prolactin production may be sustained. It may be that this pattern further evolves in different directions in different individuals as their systems respond to the impact of newly exposed, previously cryptic autoantigens or of inflammatory mediators triggered by adverse environments at the ocular surface.

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10

Antigen-Presenting Cells and Molecular Mechanisms Underlying Induction of Immune Deviation

Sharmila Masli, J. Wayne Streilein,† and A. Paiman Ghafoori

Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

INTRODUCTION

Immune privilege in the eye is known to protect the precious microanatomy of the visual axis from the inflammatory assault of an immune response, thereby avoiding any damage to accurate vision, while permitting expression of protective adaptive immunity. A fine balance between the protective and detrimental effects contributed by the immune system is maintained by the unique ocular microenvironment as well as specialized ocular cells. The peripheral adaptive immune responses to ocular antigens are directed by bone-marrow derived antigen-presenting cells (APCs) of the eye. Understanding mechanisms utilized by these cells to induce the unique immune response to ocular antigens is vital to the development of strategies to eliminate or avoid undesirable ocular immune responses. Analysis of such naturally existing mechanisms that avoid damaging immune responses also offers the opportunity to apply these mechanisms to generate therapeutic approaches to prevent inflammatory disease processes in non-immune privilege parts. Our current understanding of the mechanisms underlying antigen presentation as it relates to the induction of immune deviation is presented below.

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†Deceased.

IMMUNE RESPONSE TO OCULAR ANTIGENS

Although many local factors contribute to a fine balance maintained in the eye between prevention of inflammation and promotion of immune protection, anterior chamber associated immune deviation (ACAID) represents an active phenomenon that induces a systemic effect that is involved in maintaining the immune-privileged status of the eye. Antigens introduced in the anterior chamber of an eye invoke a unique systemic immune response that is distinct from a conventional immune response. This immune response to ocular antigens differs from a conventional response in that it is deficient in pro-inflammatory (Th1) effectors and complement fixing antibodies (IgG2a) (1) that are potentially detrimental to the ocular tissue.

The systemic nature of ACAID was originally demonstrated when inoculation of F1 hybrid lymphocytes in the anterior chamber of parental strain recipients led to systemic suppression of cell-mediated immunity that allowed prolonged acceptance of orthotopic skin allografts (2). Similarly, tumor cells bearing minor antigens different from those of the recipient when injected into the anterior chamber prevented subsequent rejection of a skin graft expressing those minor antigens (3,4). This failure to reject allografts correlated with the absence of alloantigen-specific delayed type hypersensitivity (DTH) response. Such immune deviation was also transferable to naïve recipients via adoptive transfer of spleen cells (5). This systemic effect was antigen specific. Paradoxically, presence of alloantibodies and alloantigen-specific cytotoxic T cells further underscored the uniqueness of the systemic immune response to eye-derived antigens. More recently it was demonstrated that when a soluble antigen such as ovalbumin is injected in the anterior chamber of an eye, ovalbumin-specific cytotoxic cells are inhibited (6).

EYE-DERIVED APCs

During the analysis of mechanisms responsible for the immune privilege property of the eye it was determined that bone marrow-derived cells in the iris and ciliary body of the anterior chamber exhibit immunoregulatory properties in that these cells not only failed to stimulate allogeneic lymphocytes but also suppressed mixed lymphocyte reaction (7). These bone marrow-derived cells predominantly expressed F4/80 marker and about one third of these cells expressed CD11b/Mac-1. Detection of such cells expressing markers typically present on macrophages suggested a possibility of their role in antigen presentation that results in a unique immune response to antigens introduced in the eye. Further, in the absence of lymphatic drainage of the eye, antigen-bearing cells were postulated to leave the eye via the blood to induce a systemic immune response. Accordingly, antigen-specific cells capable of inducing immune deviation were detected in the peripheral blood of animals receiving that antigen in their anterior chamber (8). These cells expressed F4/80 and were believed likely to be eye-derived since antigen introduced at sites other than anterior chamber did not release such cells into the

peripheral circulation. Presence of cells capable of transferring immune deviation in the spleens of mice receiving anterior chamber inoculation of an antigen suggested the spleen to be the likely destination of the F4/80 expressing cells that exited the eye. While F4/80-expressing cells derived from the peritoneal cavity were found to uniquely localize to the spleen when treated with ocular tissue-derived factors, more recently such cells were reported in the marginal zone of the spleen in aggregates of T and NKT cells (9). Thus, local F4/80 expressing APCs of the iris and ciliary body are believed to carry antigen via the blood to the spleen where their interactions with lymphocytes leads to the generation of regulatory cells that actively maintain the unique peripheral response to antigens introduced in the eye. Recently, the long-held view of a lack of lymphatic drainage in the eye has been re-evaluated and the existence of lymphatic drainage available to ocular antigens was documented (10). Moreover, tracking of fluorescently labeled antigens introduced in the anterior chamber of an eye revealed the presence of this antigen in the secondary lymphoid organs such as the submandibular lymph nodes and cervical lymph nodes as well as the spleen. The antigen-bearing cells were predominantly macrophages.

Similar to resident ocular F4/80$^+$ cells from the normal iris and ciliary body, extraocular F4/80$^+$ macrophages acquire the ability to induce immune deviation when exposed to the ocular environment upon their injection into the anterior chamber (11). Such acquisition of this unique ability to induce immune deviation is also possible in vitro by exposure of F4/80$^+$ macrophages to aqueous humor or culture supernatants from cells largely responsible for aqueous humor production, i.e., iris and ciliary body (12). The ability to alter the functional phenotype of F4/80$^+$ macrophages is attributed to intraocular TGFβ. This well-known immunosuppressive cytokine is present in abundance in the aqueous humor and is also produced by the parenchymal cells of the iris and ciliary body.

CHARACTERIZATION OF EYE-DERIVED APCs

In vitro exposure of F4/80$^+$ cells from the peritoneal exudate to TGFβ renders them capable of inducing immune deviation similar to that induced by eye-derived APCs (13). Such TGFβ-treated peritoneal exudates cells (PECs) when pulsed with heterogeneous antigens such as ovalbumin (OVA) can stimulate OVA-specific T cells in vitro in a manner that prevents the synthesis of inflammatory cytokines such as IFN-γ, thereby disabling their Th1 type pro-inflammatory activity (14). Further, these T cells exhibit properties similar to those expressed by regulatory T cells detected in the spleens of mice that receive anterior chamber inoculation of the antigen (15). Similar to in vivo generated regulatory cells, T cells co-cultured with TGFβ-treated APCs suppress both the induction and expression of delayed type hypersensitivity response (15,16). Therefore, analysis of TGFβ-treated APCs provided insights into mechanisms utilized by eye-derived APCs during antigen presentation. Exposure to TGFβ impairs the ability of APCs to express accessory molecules (IL-12, CD40) important in the induction of a

conventional immune response (17). These APCs begin to synthesize other immunomodulatory cytokines such as IL-10 and type I IFN (18,19). Also, TGFβ-exposed APCs produce increased amounts of active TGFβ, which in turn can influence APCs in an autocrine or paracrine manner and allow amplification of the original TGFβ effect (14). Expression of chemokines such as MIP-2 was also found to be increased in TGFβ-treated APCs (9,19). It was further determined that this chemokine permits recruitment of innate cells such as NKT cells to the marginal zone of the spleen where APCs present antigens to T cells and are engaged in inducing a regulatory cell population that imparts the systemic effect resembling peripheral tolerance (20). Along with marginal zone B cells and NKT cells, TGFβ-treated APCs create a microenvironment that is conducive to the generation of regulatory T cells. This microenvironment is rich in immunosuppressive cytokines such as IL-10 and TGFβ. Thus, ocular APCs are believed to create a TGFβ-rich environment away from their origin and allow the generation of regulatory cells.

MOLECULAR MECHANISMS UNDERLYING ANTIGEN PRESENTATION BY EYE-DERIVED APCs

Impaired expression of IL-12 has become a prototypic property of tolerogenic APCs. Functional characteristics of TGFβ-treated APCs are consistent with such tolerogenic APCs because TGFβ exposure of conventional APCs confers upon them the ability to generate regulatory cells that suppress systemic Th1-mediated immune responses, such as DTH. The absence of a pro-inflammatory cytokine such as IL-12 appears to be critical in the induction of immune deviation by eye-derived APCs in that its absence aborts differentiation of antigen-specific T cells activated by these APCs down the Th1 pathway (21). Development of Th1 effectors is restored by the addition of exogenous IL-12 in the anterior chamber along with the antigen. Therefore TGFβ exposure of APCs is likely to induce pathways that downregulate their IL-12 expression, which in turn contributes to their unique ability to induce immune deviation. In TGFβ-treated APCs, one indicator of such a possibility of IL-12 regulation was their decreased expression of CD40, a molecule known to induce IL-12 synthesis upon its ligation by corresponding CD40L on activated T cells.

Comparison of the transcriptional programs of APCs exposed to TGFβ with that of untreated APCs by microarray analysis offered an opportunity to examine candidate molecules that support the ability of APCs to induce immune deviation. Such analysis revealed increased expression of molecules that contribute to downregulation of IL-12. These included thrombospondin (TSP), TNFR II(p75), and IκBα (19). To analyze the significance of these molecules as it relates to the induction of immune deviation, we assessed involvement of these molecules in the regulation of IL-12 synthesis and subsequent suppression of DTH response by TGFβ-treated APCs.

Thrombospondin

Of the five known isoforms of this extracellular matrix (ECM) protein, TSP1 synthesis was upregulated in TGFβ-treated APCs. This ECM protein is a large (450 kD) molecule with multiple functional domains that allow its binding to various receptors such as CD47, CD36, αvβ3, heparan sulfate, and integrins (22). Such ability to bind different receptors provides a functional diversity to TSP1 that depends on the effect of its binding to these receptors on various cells and subsequent signaling induced within these cells. For instance, the anti-angiogenic effect of TSP1 is attributed to its ability to bind CD36 on vascular endothelium and the resulting apoptosis of these cells (23,24). The extensively analyzed property of TSP1 to activate latent TGFβ has been associated with its binding of CD36 on macrophages (25). More recently, ligation of CD47 by TSP1 on monocytes and macrophages was reported to inhibit secretion of IL-12 (26,27). Similarly, such CD47 ligation was reported to prevent maturation of dendritic cells as well as block their ability to generate Th1 effectors (28). Consistent with these observations, TSP1 in TGFβ-treated APCs contributes to both the activation of latent TGFβ as well as regulation of IL-12, as APCs are known to express both CD36 and CD47 (29). Ligation of CD47 on T cells has been demonstrated to induce signals that influence TCR-mediated signaling events and therefore are known to alter T cell activation (30,31). It was also proposed that by binding CD47 and CD36 on different cells TSP could provide a trimolecular bridge between those cells. Therefore, it appears possible that TGFβ-treated APCs utilize a multifunctional molecule such as TSP to tether latent TGFβ on their cell surface via their CD36 receptor such that active TGFβ is made available in the microenvironment. Thrombospondin may also regulate their IL-12 secretion via CD47 ligation, and, furthermore, APCs may also use CD36 bound TSP to bind CD47 on effector T cells, influencing their TCR mediated signals in a way that leads to generation of regulatory cells rather than Th1 type. In the absence of TSP, APCs treated with TGFβ failed to induce immune deviation (29). Therefore, TSP-mediated molecular mechanisms employed by eye-derived APCs are critical for their ability to induce immune deviation.

TNFR II (p75)

TGFβ-treated APCs increase their expression of TNFR II. These APCs also synthesize and release increased levels of TNF-α32. Contrary to its traditional pro-inflammatory role, TNF-α is essential for the induction of immune deviation as anti-TNF-α antibodies injected at the time of anterior chamber inoculation of an antigen or after intravenous injection of antigen-pulsed TGFβ-treated APCs prevents suppression of the DTH response (32,33). Such an anti-inflammatory role of TNF-α was originally suggested in TNF-α–deficient mice, as their homeostatic regulation of inflammation was impaired (34). In these mice a role for TNF-α in limiting the inflammatory response was implicated. Such a role was later

demonstrated to be effective through TNF-α–mediated regulation of IFN-γ–induced IL-12 secretion in macrophages (35). Similarly, it is quite likely that TNF-α released by TGFβ-treated APCs contributes to its impaired ability to secrete IL-12. The inability of TNF-α deficient APCs to induce immune deviation or inhibit IL-12 secretion after their TGFβ treatment supported such a possibility (36). The pro-inflammatory effects of TNF-α are primarily associated with signals mediated via TNFR I (p55); however, whether TNFR II (p75) is likely to induce anti-inflammatory signals is not yet clear (37). The difference between the two TNF receptors in their affinity for TNF-α is well established. It is also reported that the receptor with higher affinity for TNF-α, i.e.,TNFR II, is inefficient at activating the pro-inflammatory transcription factor NFκB as compared to TNFR I (38). Such a difference raises the possibility that signals mediated through TNFR II may contribute to anti-inflammatory effects. This possibility is further strengthened by increased expression of TNFR II in TGFβ-treated APCs that are capable of suppressing inflammatory DTH response. This increase in the TNFR II is significant to their immune deviation-inducing property as its absence prevents APCs from undergoing functional transformation in response to TGFβ (36). Assessment of the significance of this TNFR II in the ability of TGFβ-treated APCs to induce immune deviation indicates that TNF-α synthesized by these APCs contributes to regulation of IL-12 via TNFR II and thereby exerts its anti-inflammatory effect.

IκBα

Transcription factor NFκB proteins are present in the cytoplasm associated with the inhibitory IκB proteins. Binding of IκB proteins is known to mask the nuclear localization signal (NLS) of NFκB proteins thereby preventing their access to the nucleus (39). Phosphorylation of IκB proteins initiates their dissociation and degradation, allowing nuclear translocation of NFκB proteins. In the nucleus NFκB proteins bind their cognate DNA binding sites to regulate transcription of a large number of genes that include pro-inflammatory molecules such as CD40 and IL-12. Therefore, activation of the NFκB pathway is associated with inflammatory processes. In dendritic cells it was also demonstrated that antigen presentation is dependent on NFκB activation and that different aspects of this process, such as MHC and co-stimulatory molecule expression, as well as cytokine production, are coordinately regulated (40,41). Inhibitory IκB proteins are known to interrupt the NFκB pathway, and therefore inflammatory signals, by avoiding the NFκB-mediated transcription of genes. This is accomplished by either retaining NFκB proteins in the cytoplasm or by dissociating DNA-bound NFκB in the nucleus. Newly synthesized IκBα proteins have an intrinsic NLS that allows their entry into the nucleus, displacement of NFκB from its DNA binding sites, and transport of NFκB back to the cytoplasm (39). Increased expression of IκBα in TGFβ-treated APCs is consistent with their impaired expression of NFκB-regulated pro-inflammatory molecules such as CD40 and IL-12. Therefore, IL-12 synthesis in TGFβ exposed APCs is likely to be impaired due to inhibition of a transcription factor, NFκB, associated with its synthesis. Blocking synthesis of

IκBα in TGFβ-treated APCs using an RNA interference strategy allowed us to examine the significance of this regulatory protein in the induction of immune deviation (42). Results from our studies allowed us to conclude that TGFβ exposure of APCs prevents transcription of pro-inflammatory genes, presumably by inhibiting the transcriptional activity of NFκB.

SUMMARY

The unique immune response to eye-derived antigens is attributed to functionally specialized resident APCs. These APCs induce immune deviation, a form of peripheral tolerance, which contributes to the immune privilege status of the eye by generating regulatory cells in a secondary lymphoid organ such as the spleen. TGFβ in the ocular microenvironment confers the ability to induce immune deviation on APCs. These APCs suppress peripheral Th1-mediated immune responses such as DTH, which is accomplished by multiple molecular mechanisms invoked under the influence of TGFβ. By lowering their expression of pro-inflammatory molecules such as CD40 and IL-12, these APCs avoid inflammatory immune responses while increased expression of molecules such as thrombospondin, TNFR II, and IκBα contributes to anti-inflammatory effects by helping maintain lowered IL-12 expression. Thrombospondin also allows activation of latent TGFβ tethered to the cell surface, thereby providing an immunosuppressive microenvironment that resembles the ocular microenvironment. These mechanisms allow eye-derived APCs to avoid pro-inflammatory effects while promoting anti-inflammatory effects giving rise to immune deviation.

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11

Regulatory Dendritic Cells and Their Potential for Tolerance Induction

Audrey H. Lau and Angus W. Thomson
Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

INTRODUCTION

Dendritic cells (DCs) are bone marrow (BM)-derived professional antigen (Ag) presenting cells (APCs) that take up, process, and display Ag for recognition by lymphocytes. Upon migration from blood into peripheral tissue, interstitial immature DCs survey the microenvironment by ingesting surrounding cell products and extracellular fluid by phagocytosis and macropinocytosis. When they encounter foreign Ags, including products of microbial and viral pathogens, DCs rapidly undergo maturation and acquire enhanced ability to migrate via lymph to draining lymph nodes. Research on these highly specialized APCs has shown that they have the potential to induce tolerance (i.e., with respect to self-Ag and in experimental autoimmune disease or transplantation models) as well as augment specific immune responses (i.e., tumor immunity and resistance to infectious agents). These divergent properties of DCs can be traced to the procedures by which they are isolated, propagated, or altered and to their state of maturation. Here, we concentrate on the tolerance-inducing properties of DCs and on how these can be promoted in vitro or in vivo for potential therapeutic application.

DCs AND PERIPHERAL TOLERANCE IN THE STEADY STATE

Once immature DCs have taken up residency in peripheral tissue, they constantly survey their microenvironment through vesicular exchange with other resident

cells and the uptake of apoptotic cells and soluble material. Even in the absence of stimulation via pathogen products, other Toll-like receptor (TLR) ligands, or alloAg, DCs constantly migrate from peripheral tissue sites via the lymphatics to draining lymphoid tissue. These DCs may express self-peptide in the context of self-major histocompatibility complex (MHC) gene products. While they are generally immature, expressing low levels of surface MHC and costimulatory molecules, they may also be considered semi-mature if they express moderate levels of T cell costimulatory molecules (in particular, CD80 and CD86). It is thought that once these DCs expressing self-peptide reach secondary lymphoid tissue, they play a role in maintenance of peripheral T-cell tolerance via one or more mechanisms that include deletion of Ag-specific T cells via apoptosis, induction of T-cell anergy resulting from Ag presentation without appropriate costimulation, and the generation of T regulatory cells (Tregs) that actively suppress T-cell responses.

Oral tolerance is a prime example of how the normal steady state of the immune system within the gut and liver is associated with the generation of peripheral T-cell tolerance. It has been shown that consumption of large doses of oral Ag in experimental animals can lead to apoptosis of Ag-specific T cells (1). In separate experiments, transgenic animals with large numbers of T cells expressing a T-cell receptor (TCR) specific to ovalbumin were fed the Ag. These TCR-expressing T cells could be detected after feeding, but were refractory to further stimulation with Ag (2). Moreover, transfer of Ag-specific Tregs into Ag-naïve mice inhibited the responses of the naïve T cells to Ag in the recipient animals (3). In the context of oral tolerance, DCs in intestinal Peyer's patches express IL-10 and IL-4, in contrast to IFN-γ and IL-12 expression by DCs in peripheral lymphoid tissue (4). Further, studies with rat intestinal lymph-borne DCs (IL-DCs) have suggested a role for these APCs in promoting self-tolerance (5). These IL-DCs were not only weak APCs for induction of Ag specific and allogeneic T-cell proliferation, but they have been shown to transport apoptotic intestinal epithelial cells to T-cell areas of draining lymph nodes, a process speculated to be associated with promotion of T-cell tolerance. Together, these studies provide evidence for a role for DCs in peripheral tolerance.

DCs AND INDUCTION OF ADAPTIVE IMMUNE RESPONSES

When the normal steady state is disturbed by the presence of foreign Ag, i.e., pathogens and their products or donor alloAg, immature DCs begin to mature [i.e., upregulate surface costimulatory molecules and appropriate chemokine receptors (i.e., CCR7)] and exhibit enhanced migratory responses to secondary lymphoid tissue. When these DCs enter the lymph nodes, they interact with T cells and induce their activation. The manner by which DCs are activated, combined with the subsequent response of these cells (i.e., upregulation of costimulatory molecules and cytokine production), both contribute to the type of T-cell response induced.

In the case of organ transplantation, the recipient's T-cell responses can be induced and affected via two distinct pathways (6). Donor tissue contains interstitial DCs, which can directly activate recipient T cells (direct pathway). In this scenario, donor DCs present alloMHC to specific alloreactive recipient T cells, which respond in a vigorous manner. Alternatively, recipient T cells can be activated against donor Ag through the indirect pathway. In this instance, recipient DCs process and present donor alloAg in the context of self-MHC to recipient T cells, which are then induced to proliferate.

Autoimmunity occurs when the host's immune system responds inappropriately to self-Ag. There is evidence that DCs play a major role in the development and/or course of certain autoimmune diseases. Experimental autoimmune encephalomyelitis (EAE) is a T-cell mediated inflammatory central nervous system (CNS) disease model of multiple sclerosis. EAE is induced by immunizing C57BL/6 mice with CNS-specific myelin proteins such as myelin-basic protein (MBP). Matyszak and Perry (7) first described the presence of DCs in the brains of rats with EAE. They found increased numbers of DCs in close contact with lymphocytes in the delayed hypersensitivity EAE model and hypothesized a role for DCs in the chronicity of the disease. More recent studies on DCs isolated from brains of EAE-stricken mice revealed a similar phenotype to immature BM-derived DCs (BMDCs) or splenic DCs (8). However, these DCs were incapable of priming naïve T cells and, further, they inhibited T-cell proliferation stimulated by mature BMDCs. Thus, data on CNS-DCs are conflicting and suggest a potential role for these DCs in either the course of autoimmune disease or in the maintenance of immune privilege in the CNS.

DCs AND TOLERANCE INDUCTION

With improved understanding of DC biology and of the role of DCs in peripheral tolerance, as well as advances in techniques to isolate and generate large numbers of DCs, it has become increasingly likely that DCs can be used as therapy to induce tolerance. Various types of DCs, isolated and generated under different conditions, have been examined for their potential tolerogenic/regulatory properties. Rather than activating T cells to skew to either T helper (Th) 1 or Th2 cell production or to induce cytotoxic T lymphocytes (CTLs), these tolerogenic DCs would instead promote anergy or apoptosis of reactive Th1/Th2 cells, induce Tregs, or decrease the induction of CTLs. The following approaches have been used to obtain such tolerogenic DCs: (*i*) fresh isolation of (immature) DCs, (*ii*) specific culture conditions, (*iii*) pharmacological treatment of the DCs, and (*iv*) genetic engineering.

Freshly Isolated DCs (Immature DCs or DC Progenitors)

$CD11c^+$ DCs of phenotypically distinct subsets have been described and isolated from various murine tissues (9–17). These include the classic myeloid DCs ($CD11b^+CD8\alpha^-$), "lymphoid-related" DCs ($CD11b^-CD8\alpha^+$), and more recently,

Table 1 Review Articles on Regulatory Dendritic Cells

Type of modification	Reference
General reviews; multiple methods discussed	6,20–25
Immunosuppressive drugs	26–28
Gene therapy	29,30

plasmacytoid DCs (pDCs; $B220^+$). When $CD11c^+$ DCs are freshly isolated from normal lymphoid or non-lymphoid tissues (such as spleen or liver), they are immature in cell surface phenotype, as characterized by low CD40, CD80, CD86, and MHC II expression. Such immature DCs of donor origin, when infused prior to transplantation, prolong organ (18) or pancreatic islet (19) allograft survival, without use of immunosuppressive therapy. The mechanisms by which these freshly isolated DCs promote tolerance when administered in experimental transplant models have not been fully elucidated. Hypotheses to explain this phenomenon include induction of T-cell anergy/apoptosis by allogeneic DCs expressing low or no levels of classical costimulatory molecules (signal 2), or induction of Tregs capable of suppressing alloreactive T-cell responses (Table 1).

The classic mechanism by which T-cell anergy is induced is Ag presentation (signal 1) without appropriate costimulation (signal 2). In studies using freshly isolated DCs to promote Ag-specific tolerance in transplant recipients, it has been argued that this mechanism may play a major role as immature DCs, lacking CD80 and CD86, constitute a major source of alloAg. O'Connell et al. (31) found that, in a murine allogeneic cardiac transplant model, administration of either immature or mature splenic $CD8\alpha^+$ DCs of donor origin significantly prolonged transplant survival. Furthermore, they showed that prolongation of allograft survival was not dependent upon a specific subset of immature DCs (31). Thus, both immature classic $CD8\alpha^-$ and $CD8\alpha^+$ DCs prolonged graft survival. In the context of allergic hypersensitivity, Akbari et al. (32) have shown that pulmonary DCs have a role in the induction of tolerance. When mice were exposed to inhaled nonpathogenic Ag, pulmonary DCs from these mice were phenotypically mature, yet transiently produced IL-10. They argued that production of IL-10 by pulmonary DCs was critical for the induction of Ag-specific tolerance.

In organ transplantation, the ability of immature donor DCs to promote tolerance can be enhanced by simultaneous administration of other tolerance-promoting agents, such as anti-CD154 (CD40L) monoclonal antibody (33) (mAb) or the costimulation blocking agent (fusion protein) cytotoxic T-lymphocyte antigen-4 (CTLA4)-Ig (34). When these agents are combined with the DCs, graft survival can be markedly extended. Anti-CD154, which blocks the CD40-CD154 pathway, has been found to prolong graft survival when used therapeutically in experimental transplant models. Wang et al. (35) have used an aortic allograft model to show that when anti-CD154 is administered in combination with freshly isolated immature donor DCs, transplant vascular sclerosis is markedly reduced.

Additional data show that either alone or in combination with modified DCs, anti-CD154 prolongs graft survival either in rodent or non-human primate transplant models (33,36–39). CTLA4-Ig binds to the costimulatory molecules CD80 and CD86 on DCs, preventing their interaction with CD28 and CTLA4 on T cells. This costimulation blockade prolongs graft survival in rodent studies (40–43). When rats were pretreated with both dexamethasone-treated allogeneic F1 DCs and CTLA4-Ig, T cells were rendered unresponsive to indirect presentation of alloAg (host APCs presenting donor Ag to T cells) but retained their ability to respond via the direct pathway (host T cells recognize donor APC/Ag) (44). This course of therapy, together with a brief early course of cyclosporine A (CsA) immediately after transplant to inhibit the direct pathway, resulted in indefinite kidney allograft survival. While current studies of tolerogenic DCs have not yet been undertaken in non-human primates, testing of this type of DC-based therapy can readily be envisaged in a clinically-relevant model, such as the rhesus macaque, in which DC subsets have recently been characterized (45,46).

Freshly isolated DCs may also be innately different in their function depending upon their tissue of origin. For example, the liver microenvironment is high in the immunosuppressive cytokines IL-10 and TGF-β1, which may have direct inhibitory effects on DC development, maturation, and function (47). Freshly isolated DCs from the respiratory tract, intestinal Peyer's patch, and liver have been found to be poor allostimulators of T cells in mixed leukocyte reaction (MLR) (48–50). Accordingly, these DCs are poor synthesizers of bioactive IL-12p70, but exhibit high levels of expression of IL-10 mRNA or protein (48–50). DCs isolated from a specific location, such as the liver, may have the potential to further promote tolerance because of an inherent tolerogenic capacity.

In addition to potential differences in cytokine production by various tissue-resident DCs, these APCs may also be the source of other potential immunoregulatory proteins. Indeed, there may be preferential or exclusive production of specific immunoregulatory proteins by certain DCs. For example, much recent interest has focused on indoleamine-2,3-dioxygenase (IDO), which has been shown to suppress T-cell proliferation by catabolizing tryptophan upon which T cell replication is dependent. Several groups have examined the role of IDO in DCs function. Mellor et al. (51) showed recently that, in mice treated with CTLA4-Ig, splenic DCs subsets, including CD8α^+ DCs, pDCs, and bitypic natural killer/DC regulatory cells (52) (NK DCs; DX5$^+$CD11c$^+$CD8α^+), upregulated IDO production. Our laboratory has preliminary data that support the hypothesis that IDO production may be one contributing factor to tolerance induction by freshly isolated pDCs. Freshly isolated murine splenic pDCs strongly express mRNA for IDO, in contrast to myeloid and CD8α^+ DCs. Similar to other freshly isolated DCs subsets, freshly isolated pDCs of donor origin were able to significantly prolong murine cardiac allograft survival (53).

While it is known that administration of freshly isolated DCs can promote tolerance in the context of experimental transplantation, the specific mechanisms by which different DC subsets achieve this effect are not yet understood. Methods

to enhance the capacity of freshly isolated DCs to induce tolerance are under study. In particular, tissue-specific DCs are being studied for their differences in maturation and function and thus their ability to promote tolerance. Addition of other therapeutic agents, such as immunosuppressive drugs, other agents that are known to subvert DCs maturation, and those that block costimulation, in concert with freshly isolated or in vitro-propagated DCs, is under investigation.

Specifically Cultured DCs

While freshly isolated DCs have been shown to promote tolerance in experimental models, practical issues concerning their clinical use have arisen. Many studies have used BM of DCs or DCs from other tissues, especially spleen or thymus, which would not normally be available for human use. Furthermore, the timing of the isolation or propagation of these DCs must be considered in relation to when (e.g., in relation to cadaveric organ transplantation) the DCs would be needed for therapeutic administration. A growing area of interest is in culturing DCs from BM progenitors or blood-borne precursors under specific conditions that render the DCs tolerogenic.

DCs are commonly generated in bulk from BM progenitors by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF, with or without IL-4) to the culture. DCs generated in this manner are a heterogenous population of immature and semi-mature APCs. In the non-obese diabetic (NOD) mouse model of type-1 diabetes, it has been shown that administration of syngeneic BMDCs generated with GM-CSF and IL-4 to pre-diabetic mice prevented the onset of autoimmune diabetes (54). T cells from DC-treated mice were found to have Th2-like properties, such as the production of high levels of Th2 cytokines.

By adding other cytokines or stimuli to DC cultures, the phenotype and function of these cells can be altered. Lutz et al. (55) reported that murine DCs propagated from BM progenitors in GM-CSF and treated concurrently with a high dose of lipopolysaccharide (LPS) are immature and induce alloAg-specific $CD4^+$ T cell anergy in vitro. By altering the culture conditions, Sato et al. (56) have generated an alternatively-activated DC population capable of inducing tolerance in allogeneic BM transplantation. These DCs, termed regulatory DCs (rDCs), are generated with GM-CSF, IL-10, and TGF-β and stimulated with high dose LPS near the end of culture. These rDCs express very little CD80 or CD86 but high levels of MHC II. Further, these rDCs are very poor stimulators in allogeneic MLR as compared to Vitamin D_3-conditioned DCs (tolerogenic, discussed in next section). In an elegant study using a mouse model of acute graft-versus-host-disease (GVHD), Sato et al. found that pre-treatment of recipient mice with host-matched rDCs prevented acute lethal GVHD, which was Ag-specific, and complete. In studying the mechanism by which the rDCs exerted their action, $CD4^+$ T cells from rDC-treated recipients were hyporesponsive to stimulation with host-type mature DCs, while $CD8^+$ T cells had reduced lytic activity against host-matched target cells. Furthermore, the authors were able to characterize

Ag-specific IL-10-producing regulatory T cells ($CD4^+$) in protected, rDC-treated mice.

Plasmacytoid DCs can now be generated in culture from murine BM progenitors by the addition of the endogenous DC growth factor fms-like tyrosine kinase 3 ligand (Flt3L). As discussed above, in vivo-mobilized freshly isolated donor splenic pDCs can prolong graft acceptance in murine transplant models. Recent evidence suggests that cultured BM-derived pDCs, which are also immature in phenotype, can similarly prolong graft survival (57).

It has been shown that BM-derived DCs, which can be cultured in large quantities, can be modified by adjusting the culture conditions. Tolerogenic or rDCs have been generated that, in experimental animal models, can induce tolerant states. There is considerable potential for the use of this technology in developing DC-based therapy for tolerance induction, e.g., in the context of living-related organ transplantation, which would provide the necessary time for culture and administration of these cells to recipients prior to surgery.

Pharmacologically Manipulated DCs

Immunosuppressive drugs are used extensively in the treatment of various chronic inflammatory diseases, including allograft rejection. The influence of these drugs on DC development, maturation, and function is under extensive study. There is strong evidence that these and other pharmacologic agents may affect DCs. Several drugs have been studied in detail (i.e., CsA, corticosteroids) while some newer drugs (i.e., the deoxyspergualin derivative LF15-0915) have inhibitory effects on DC maturation, which may have therapeutic implications. The influence of these drugs on DCs provides potential mechanisms by which DC–T-cell interactions can be manipulated to generate T-cell unresponsiveness or desired regulation. Herein, we focus on several drugs in greater detail. These include CsA, rapamycin, dexamethasone, LF15-0915, mycophenolate mofetil, and 1α, 25-dihydroxyvitamin D_3.

Cyclosporine A

This cyclophilin ligand immunosuppressant has been in clinical use for more than two decades and is one of the most widely studied anti-rejection agents. Cyclosporine A (CsA) and tacrolimus (FK506) are pro-drugs. Once CsA or FK506 have bound their respective receptors [intracellular immunophilin cyclophilin A and FK506 binding protein 12 (FKBP12), respectively], the complex binds to calcineurin, causing inhibition of T-cell receptor (TCR)-mediated signal transduction pathways. Their primary action is therefore direct suppression of T-cell activation. Both of these immunosuppressants inhibit DC maturation, characterized by downregulation of costimulatory molecule expression, poor allostimulatory capacity, and inhibited production of Th1 and Th2 cytokines (58–60).

It has been shown that CsA administration impairs the function of DCs and epidermal Langerhans cells (LCs). Knight et al. (61) found that DCs isolated from the lymph nodes of CsA-treated mice had lower immunostimulatory capacity as well as low levels of Ag presentation capacity in comparison to those from untreated animals. This difference was hypothesized to be due to CsA affecting the acquisition and/or presentation of Ag by DCs. Further investigation revealed that splenic DCs were also sensitive to CsA administration and were more sensitive to low doses of CsA than lymphocytes (62). It was also found that CsA impaired the accessory cell function of murine and human LCs in vitro and that this was not dependent upon IL-1 or prostaglandin production, or dependent on changes in MHC class II expression (63,64). However, the Ag-presenting capacity of LCs is inhibited by CsA (63–65).

Lee et al. (66) showed that CsA inhibited the allostimulatory capacity of in vitro-generated murine BM-derived DCs by downregulation of surface costimulatory molecules and that nuclear factor (NF)-κB was the molecular target in CsA's inhibitory effects on DCs. The finding of CsA's actions on costimulatory molecule expression was confirmed in a study of CsA treatment on LCs (67) as well as subsequent studies on human monocyte-derived and peripheral blood DCs (68,69).

It has been speculated that another mechanism by which CsA may exert its effects is by directly influencing the number of DCs. However, while the number and differentiation of circulating and tissue-resident DCs in humans do not appear to be affected by systemic administration of CsA (68,70), specific DC subsets may be more sensitive to CsA treatment. In the rat, the number of thymic DCs is decreased with CsA treatment (71) although the DCs present are identical in phenotype and function to control DCs (71,72). Further, studies of epidermal LC precursors have shown that CsA negatively influences their differentiation (73).

Matsue et al. (59) reported recently that CsA blocked bi-directional DC–T-cell interaction following Ag presentation. CsA effectively inhibited T-cell production of interferon (IFN)-γ, IL-2, and IL-4 triggered by murine BMDCs, as well as blocked BMDC production of IL-6, IL-12p40, and IL-12p70. CsA effectively suppressed T-cell proliferation when stimulated by DCs as well as inhibited cytokine production by both Th1 and Th2 cells. However, these CsA effects were abrogated when BMDCs were stimulated with LPS. Recent findings by Chen et al. (74) show that CsA treatment reduces CCR7 expression, contributing to impaired DCs migration to secondary lymphoid tissue. Woltmann et al. (70) reported that human monocyte-derived DCs treated with CsA have reduced production of tumor necrosis factor (TNF)-α upon stimulation. In a separate study with human peripheral blood DCs, Tajima et al. (69) showed that CsA treatment had differential effects on CD11c$^+$ (myeloid) and CD11c$^-$ (plasmacytoid) subsets. When the CsA-treated CD11c$^+$ myeloid subset was stimulated with LPS, IL-12 production was inhibited and IL-10 production augmented in comparison to control cells, while the CsA-treated, Sendai virus-infected CD11c$^-$ DCs had reduced

IFN-α production. In general, the effects of CsA on cytokine production by DCs appear to be dependent upon the stimuli used to trigger DCs maturation.

Rapamycin

Although it also binds FKBP12, rapamycin (RAPA; sirolimus) does not have the same mechanism of action as tacrolimus. Instead of inhibiting calcineurin activation, the RAPA-FKBP12 complex inhibits the mammalian target of RAPA (mTOR), a common effector protein which is critical in multiple biochemical pathways, such as cytokine and growth factor-induced proliferation, cell cycle progression into S phase, ribosomal protein synthesis, and translation initiation (75,76). The many investigations into the effects of RAPA on T cells have led to the view that T cells are the principal therapeutic target of RAPA. It has been shown in vitro and in vivo that RAPA treatment causes DCs of the same defects observed in DCs treated with calcineurin inhibitors, as well as suppression of functional activation, inhibited endocytosis, and inhibited generation and maturation of DCs from BM cultures (59,77,78).

Hackstein et al. (79) reported that, at clinically relevant concentrations, RAPA inhibited endocytosis by BMDCs both in vitro and in vivo, as well as suppressed the functional activation of BMDCs. Further studies by this group also showed that the inhibitory effects of RAPA on DCs maturation were IL-4-dependent and antagonized by competitive binding of FKBP12. When administered in vivo, RAPA impaired DC generation, costimulatory molecule expression, IL-12 production, and T-cell allostimulatory capacity (77). DCs from RAPA-treated donor animals induce alloAg-specific T-cell hyporesponsiveness in normal recipients. Chiang et al. (78) found that these RAPA-treated BMDCs also impaired induction of cytotoxic T-cell activity and had decreased Stat4-dependent IFN-γ production. Further, pre-treatment of recipients with donor RAPA-BMDCs significantly prolonged murine heart allograft survival in comparison to untreated DC-injected controls. Studies of RAPA treatment of human monocyte-derived DCs and DCs derived from CD34$^+$ precursors have reported similar effects, including impaired receptor-mediated endocytosis and decreased allostimulatory capacity, costimulatory molecule expression, and IL-12 and IL-10 production (80,81).

Dexamethasone

Glucocorticoids (GCs) act by inhibiting gene transcription by directly competing for DNA binding sites in the promoter regions or complexing with transcription factors. GCs have been shown to reduce murine splenic DC viability, downregulate their ability to express costimulatory molecules, and impair their capacity to stimulate allogeneic T cells. Dexamethasone (Dex) inhibits the early stages of TCR signaling by altering the compartmentalization of key signal transducers (82). Dex causes downregulation of costimulatory molecules, impaired Ag presentation, and reduced allostimulatory capacity of DCs (59,83,84). Further, Matyszak et al. (84) reported that Dex-treated BMDCs were unable to prime Th1 cells efficiently and

that multiple restimulation of T cells with Dex-treated BMDCs gave rise to IL-10–producing Tregs. Dex had similar effects on murine and human LCs (85–87).

Dex has been reported to impair the differentiation (70,88) and function (84,89) of human monocyte-derived DCs, although these data are debated. Other reports find that Dex does not affect T-cell proliferation (90,91). Cytokine production by monocyte-derived DCs is also debated. It has been reported that the production of IL-12p70, IL-6 and TNF-α by DCs is decreased by treatment with GCs (70,88,90–93). Other authors have additionally shown that IL-10 is increased in DCs treated with GCs (91,94).

Mycophenolate Mofetil

Mycophenolate mofetil (MMF) is a pro-drug of a fermentation product of several species of *Penicillium* (95). This anti-proliferative drug's mechanism of action is as a non-competitive, reversible inhibitor of the enzyme inosine 5'-monophosphate dehydrogenase, which is important in the de novo synthesis of guanosine nucleotides (96). MMF inhibits both T- and B-cell proliferation. MMF was first described to affect murine BMDCs in a dose-dependent manner, impairing T-cell allostimulatory capacity, decreasing costimulatory molecule expression, and reducing IL-12p70 production in response to LPS stimulation (97). A similar study using human monocyte-derived DCs confirmed these results and further, Colic et al. reported that the differentiation of these DCs was inhibited by MMF treatment through induction of apoptosis (98). Gregori et al. (99) found when MMF was used in concert with 1α, 25-dihydroxyvitamin D3 [1α, 25(OH)$_2$D3, see below], tolerogenic DCs were generated that could enhance the frequency of CD4$^+$CD25$^+$ Tregs and promote transferable tolerance to murine islet allografts.

1α, 25-Hydroxyvitamin D$_3$

The biologically active form of vitamin D$_3$ is 1α, 25(OH)$_2$D$_3$ (calcitrol). This metabolite is a secosteroid hormone that not only has a role in the regulation of bone and calcium/phosphate metabolism but also has other biological activities, including modulation of immune responses. 1α, 25(OH)$_2$D$_3$ has been shown to inhibit the maturation and function of human monocyte-derived DCs both in vitro and in vivo, suppressing IL-12 secretion while enhancing IL-10 production subsequent to CD40 ligation (100). Similar inhibitory effects of 1α, 25(OH)$_2$D$_3$ on the maturation and function of murine BMDCs (101) and LCs (102) have been described. When mice are treated with MMF and 1α, 25(OH)$_2$D$_3$, tolerogenic DCs are generated which produce reduced levels of IL-12 and increased percentage of CD4$^+$ Tregs (99,103).

Deoxyspergualin and LF15-0915

Deoxyspergualin (DSG) is isolated from cultures of *Bacillus laterosporus* (104). Its use in rodent models has been reported to prolong organ allograft survival (105,106). DSG, an inhibitor of nuclear factor (NF)-κB, inhibits the maturation

of rhesus monkey DCs in vitro and in vivo, with markedly reduced numbers of mature DCs in lymph nodes of renal allografted monkeys given a tolerogenic protocol of anti-CD3 immunotoxin and DSG (107). A novel analogue of DSG, LF15-0915 (LF), has also been described as having anti-rejection properties. Several recent reports have studied LF's inhibitory effects on DC maturation (108,109), Th2 skewing (109), and regulatory $CD4^+$ T-cell generation (108,110), which, as with DSG, are regulated through the inhibition of NF-κB. In a rat transplant model, Chiffoleau et al. (110) found that LF administration led to a significant increase in percentage of $CD4^+$ Tregs which could transfer tolerance. Similarly, Min et al. (108,111) found that LF treatment in conjunction with anti-CD45RB mAb in murine transplant models led to increases in both tolerogenic (immature) DCs and Tregs.

Genetically-Engineered DCs

Genetic engineering therapy is a relatively new field that has rapidly expanded in the past decade. A logical progression for the generation of tolerogenic DCs is the use of this approach to introduce and/or overexpress potential therapeutic genes into DCs. Many candidate genes have already been expressed in DCs and analyzed for their impact on tolerance induction. These include genes encoding anti-inflammatory cytokines (i.e., IL-10 and TGF-β), costimulation-blocking agents (i.e., CTLA4-Ig), death-inducing ligands (i.e., FasL), and IDO.

Murine BMDCs retrovirally transduced with viral IL-10 have reduced capacity to stimulate allogeneic T-cell proliferation as well as cytotoxic responses (112). Portal vein infusion of IL-10-transduced BMDCs in murine renal allograft models led to prolonged graft survival in comparison to control-transduced DCs. This increased survival was further enhanced by the additional transduction of the DCs with TGF-β (113). Use of genetically modified BMDCs has also been shown to prevent or reverse the break of self-tolerance in various autoimmune diseases, such as the NOD mouse model of type-1 diabetes and murine type-II collagen-induced arthritis. Recently, Feili-Hariri et al. (114) demonstrated that administration of BMDCs infected by IL-4-encoding adenovirus was able to prevent diabetes in NOD mice with advanced insulitis. Similarly, use of adenovirus-infected, IL-4-expressing immature BMDCs in mice with established collagen-induced arthritis led to almost complete suppression of the inflammatory disease (115,116). Not only was IFN-γ production in the spleen reduced in these treated mice, but the levels of specific antibodies against collagen were also reduced.

Similar inhibition of T-cell proliferative and cytotoxic responses has been reported for stably-immature (NF-κB anti-sense treated) murine BMDCs retrovirally transduced with CTLA4-Ig (117). Analysis of the T-cell responses revealed marked inhibition of IFN-γ but significant increases in IL-4 and IL-10 production. When these CTLA4-Ig-DCs were administered prior to murine cardiac transplantation, marked prolongation of graft survival was achieved (118).

CONCLUSIONS

DCs have inherent tolerogenic properties that can be enhanced by biologic, pharmacologic, or genetic engineering approaches. Studies using these tolerogenic DCs in transplant and autoimmune disease models show that DCs can be manipulated and used as therapy for the induction of tolerance. Further work in potentiating DC tolerogenicity will likely enhance their eventual therapeutic impact.

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12

Corneal Antigen-Presenting Cells: What Have We Learned from Transplantation?

Reza Dana

Laboratory of Corneal Immunology, Schepens Eye Research Institute, Cornea Service, Massachusetts Eye and Ear Infirmary, and Department of Ophthalmology, Harvard University, Boston, Massachusetts, U.S.A.

INTRODUCTION

Antigen-presenting cells (APCs) are critical mediators for all immune-mediated disorders, since these cells are not only the "sentinels" of the immune system for detection of foreign antigens, but they also play a critical role in tolerance induction to both self and foreign antigens (1–4). Professional APCs include dendritic cells (DCs), macrophages, B cells, and epithelial Langerhans cells (LCs) (5,6). The most potent APCs in most tissues are DCs and LCs. These cells are also known to serve as the professional APC of the cornea and ocular surface (7–12), and their recruitment to the cornea has been associated with loss of "immune privilege" in the anterior segment (13), exacerbation of herpetic (14,15) and other forms of microbial keratitis, and induction and amplification of transplant immunity (16,17). The majority of our studies on corneal APCs have relied on the corneal transplant model in the mouse, and in this overview, we refer primarily to this model for delineating the major mechanisms involved in corneal APC mobilization and activation.

RECRUITMENT OF APCs INTO CORNEA

The limbus, the area between the vascularized conjunctiva and avascular cornea, has a significant resident population of MHC class II^{+} LC that when stimulated

can be recruited into the cornea (11,18–21). Our laboratory has identified interleukin-1 (IL-1), a potent pro-inflammatory cytokine, as playing a major role in the recruitment of limbal APCs into the cornea. We have shown that the early expression of IL-1 by the inflamed cornea leads to profound upregulation (even within hours) in the expression of the cell adhesion molecule ICAM-1 by the limbal vascular endothelium, which precedes the recruitment of leukocytes to the peripheral cornea (22). The role of IL-1 is confirmed in the transplant model in which the recruitment of host-derived LCs into the allografts is suppressed as a result of IL-1 blockade (23). However, IL-1 is not the sole cytokine involved in this process. Tumor necrosis factor-alpha (TNF-α) also plays an important role. Indeed, local suppression of TNF-α signaling can significantly suppress limbal LC recruitment into the cornea by effective suppression of gene transcription for RANTES and MIP-1β (24), two principal ligands of CCR5, a chemokine receptor that mediates LC recruitment into the cornea (25).

CORNEAL APCs ARE COMPRISED OF A HETEROGENEOUS POPULATION

Our data in the mouse model suggest that there are at least four distinct bone marrow-derived CD45$^+$ cells in the cornea. First, we have identified CD11c$^+$CD11b$^-$ LCs in the epithelium (including central regions) of the normal cornea that bore classic ultrastructural features of epidermal LCs (7,9). Second, both our and Hendricks' laboratories have identified CD45$^+$CD11b$^+$CD11c$^-$ monocytic cells in the corneal stroma (7,8,26). Third, in the very anterior portions of the corneal stroma, we have also identified a population of myeloid CD8α-monocytic (CD11b$^+$) CD11c$^+$ dendritic cells (8) demonstrating distinct ultrastructure as compared to the monocytic CD11c$^-$ cells present in the more posterior portions of the cornea. Finally, a fourth population of CD14$^+$ undifferentiated or precursor-type cells have been identified throughout the corneal stroma, most of which fail to express classic monocytic or dendritic cell surface markers (7). For APCs to prime T cells they need to present antigen in the groove of MHC class II, along with requisite costimulatory signaling. However, what is unique to the bone marrow-derived cells of the *central* cornea is that they are universally both MHC class II- and costimulatory factor (CD40, CD80, CD86)-negative (8–10). While highly immature APC populations have been identified in lymphoid organs and blood (2,27), no other tissue has been reported to be replete with *universally* MHC class II-negative CD45$^+$ cells.

FUNCTIONAL ASPECTS OF CORNEAL APCs

Our experimental data suggest that in addition to the recruitment of a large numbers of cells from the limbus into the cornea, there is also a profound upregulation in the expression of MHC class II and costimulatory molecules (CD40, CD80, CD86) by resident bone marrow-derived cells in response to inflammation and

transplantation (7,28). Hence, corneal bone marrow-derived cells can phenotypically mature into cells that express "classic" APC characteristics.

From a functional standpoint, our data clearly show that graft-derived cells can be detected leaving the transplant and migrating centrifugally into the recipient rim, and eventually localizing into the ipsilateral draining lymph nodes of the host—strongly suggesting that these cornea-derived cells can act as "passenger leukocytes" in the context of transplantation immunology (10). Initial clues as to the functional relevance of this traffic came when we demonstrated that disruption of the eye-lymph node axis, through surgical cervical lymphadenectomy, led to both complete abrogation of host allosensitization as well as universal and indefinite allograft survival (29,30). Second, we utilized the ELISPOT assay to measure the frequency of host T cells primed directly by graft-derived APCs. These data have confirmed that in high-risk corneal transplantation (grafted onto inflamed recipient beds), but not in low-risk grafting (placed onto normal and uninflamed host beds), there is significant induction of IL-2- and IFN-gamma-secreting directly primed $CD4^+$ T cells well before the onset of clinical rejection (31). These data are the first to illustrate the direct contribution of cornea-derived cells in generating the immune response to corneal antigens. Second, these data illustrate, for the first time, how the microenvironmental specificities of the transplant site (e.g., degree of inflammation or vascularity) can have a profound effect on the differential contribution of the distinct pathways of sensitization generated in the host in response to grafted antigens.

CORNEAL APC TRAFFICKING TO LYMPHOID TISSUES

The data summarized above suggest that corneal bone marrow-derived cells are capable of trafficking efficiently to lymphoid organs and functioning as APCs for priming T cells. But how do these cells so readily gain access to lymphoid reservoirs? The answer to this lies with signaling mediated by vascular endothelial growth factor receptor (VEGFR)-3, a receptor that is distinct from VEGFR-1 and VEGFR-2 that regulate *blood* angiogenesis (32). The ligands to VEGFR-3 are VEGF-C and VEGF-D, both of which can also serve as growth factors for lymphatic endothelium (33), and can hence promote lymphangiogenesis. We have recently discovered that VEGFR-3 over-expression by endothelial cells in response to inflammation is also accompanied by surface expression of VEGFR-3 by mature corneal DCs (34). Indeed, we have shown that in corneal inflammation, the DCs and monocytes that congregate around the budding lymphatics are nearly all VEGFR-3+, suggesting that they may respond to the same signals (e.g., VEGF-c) that induce lymphatic growth into the cornea (35). To demonstrate in vitro functionality of VEGFR-3 expression by corneal APCs, these cells were harvested from corneal explants and placed in a transwell chamber, where their dose-dependent chemotactic responsiveness to VEGF-C was confirmed (36). The in vivo relevance of VEGFR-3 ligation was tested using either a VEGFR-3/Ig chimeric molecule or an anti-VEGFR-3 antibody, whereby we showed that

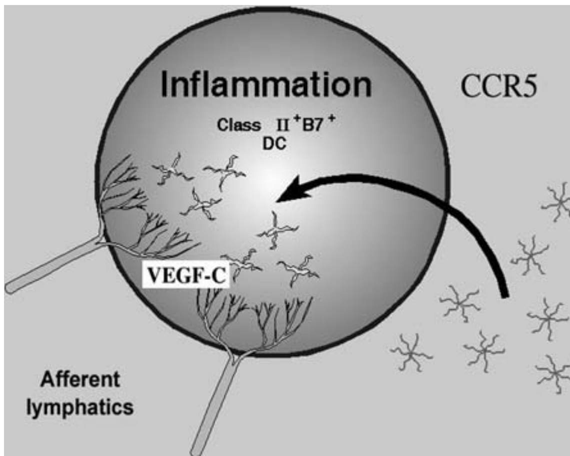

Figure 1 In response to inflammation, antigen-presenting cells (APCs) residing outside the cornea, primarily in the limbus, are recruited into the cornea through the function of CCR5 chemokine receptor. Once in the cornea, these APCs respond to VEGF-C, secreted by the lymphatic endothelium, and enter afferent lymphatics to exit the eye.

sub-conjunctival or systemic injection of these VEGFR-3 blocking agents could suppress (*i*) APC migration from the eye to regional draining lymph nodes, (*ii*) induction of anti-graft delayed-type hypersensitivity, and (*iii*) transplant rejection (36). Accordingly, we have coined the term "molecular lymphadenectomy" to connote the functional effect of VEGFR-3 antagonism as a non-surgical strategy that targets lymphatic drainage.

CONCLUSIONS

In conclusion, we have demonstrated that the normal cornea in fact possesses a heterogeneous population of resident bone marrow-derived cells, including DCs, and that these cells have the unique feature of being universally immature or of a precursor phenotype. We also have emphasized that the molecular regulation of APC *ingress* into the cornea (e.g., via CCR5) is distinct from that which dictates their *egress* from the cornea (e.g., via VEGFR-3) and entry into lymphoid tissues (see Fig. 1). Inflammation affects the functionality of these APCs by changing their surface expression of a variety of molecules (MHC class II, CD40, CD80/CD86, VEGFR-3) that alter their capacity to interact with afferent lymphatics as well as with naïve T cells in draining lymph nodes. Additionally, our data suggest that mechanisms that regulate the access of these cells to lymphoid reservoirs can be exploited as a powerful strategy to downmodulate induction of immunogenic inflammation.

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13

Therapeutic Manipulation of Ocular Antigen-Presenting Cells in Corneal Transplantation

Jerry Y. Niederkorn
Departments of Ophthalmology and Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas, U.S.A.

INTRODUCTION

Corneal transplantation is the most common and, arguably, the most successful form of solid tissue transplantation. In the United States alone, over 33,000 corneal transplants are performed each year (1). In uncomplicated cases, 90% acceptance is commonplace even though tissue typing and administration of systemic immunosuppressive drugs are not employed. Such success is unparalleled in other forms of transplantation and has led to the proposition that corneal allografts enjoy immune privilege (2–5). A panoply of anatomic, physiologic, and immunoregulatory parameters contribute to the immune privilege of corneal allografts (Table 1). These parameters can be reduced to three broad categories: (*i*) those that block induction of alloimmunity; (*ii*) those that divert or suppress alloimmune responses; and (*iii*) those that inhibit the host's immune effector elements.

The most appealing and simple explanation for the immune privilege of corneal allografts is based on the remarkable avascular nature of the corneal graft and graft bed. The absence of lymph and blood vessels is believed to prevent the egression of alloantigens from the graft to the regional lymphoid apparatus. Indeed, it is well-recognized that corneal grafts transplanted into vascularized

Table 1 Factors Contributing to the Immune Privilege of Corneal Allografts

Factor	Effect
Avascular graft bed	Block induction of immunity
Absence of donor LCs	Block induction of immunity
Low expression of MHC antigens	Reduce immunogenicity and antigenicity
Expression of FasL on cornea	Deletion of infiltrating alloreactive lymphocytes
Anti-inflammatory and immunosuppressive factors in aqueous humor	Reduction and depletion of alloreactive lymphocytes attacking the endothelium of the corneal allograft
Complement regulatory proteins on corneal cells and in aqueous humor	Prevent expression of complement-mediated injury elicited by antibodies directed against donor alloantigens

Abbreviations: LCs, Langerhans cells; MHC, major histocompatibility complex.

graft beds are invariably rejected (2). However, the avascularity of the corneal graft bed alone cannot explain the immune privilege of corneal allografts because corneal allografts transplanted into clear graft beds can undergo immune rejection. A considerable body of evidence indicates that other features of the corneal allograft can have a major impact on the induction of alloimmunity and the provocation of corneal graft rejection. Among these is the presence of donor antigen presenting cells (APCs), namely Langerhans cells (LCs), which are capable of emigrating from the corneal graft and sensitizing the host to the corneal alloantigens. In addition to donor-derived antigen presenting "passenger cells" residing in the corneal allograft, the corneal graft bed itself contains host APCs, such as the LCs in the limbus and macrophages in the conjunctiva. These three populations of APCs have the capacity to induce alloimmune responses to corneal allografts and as such are potential therapeutic targets for promoting corneal allograft survival.

Under normal circumstances, the central corneal epithelium is devoid of dendritic APCs that constitutively express major histocompatibility complex (MHC) class II antigens (2–5). However, a variety of stimuli can induce the centripetal migration of MHC class II$^+$ APCs from the limbus to the central corneal epithelium (6–9). Recently, new light has been shed on the ontogeny and behavior of corneal APCs. Hamrah et al. demonstrated the presence of dendritic cells bearing some of the characteristic markers of LCs, such as CD11c, but failing to constitutively express MHC class II molecules (10). During corneal inflammation, these putative immature LCs undergo maturation and subsequently express MHC class II and B7 molecules (11). The cornea also contains a unique population of pleomorphic CD45$^+$ cells that reside in the central and pericentral regions of the stroma (12). These cells co-express CD11b and F4/80, suggesting that they are macrophages. However, the function of these cells remains unknown.

EFFECT OF DONOR-DERIVED "PASSENGER" LCs ON CORNEAL ALLOGRAFT SURVIVAL

Unlike the skin, which has a contiguous network of LCs, the uninflamed corneal epithelium lacks mature, MHC class II$^+$ LCs. LCs are potent activators of alloimmune responses; as few as 10 allogeneic LCs can induce allospecific, cytotoxic T lymphocyte responses (13). The central portion of the cornea that is normally used for corneal transplantation is conspicuously devoid of dendritic cells that constitutively express MHC class II and B7 molecules that are characteristic of mature LCs (2). A wide variety of stimuli induce the appearance of MHC class II$^+$ LCs in the central corneal epithelium (6–9). The presence of donor-derived LCs has a profound effect on the immunogenicity and survival of corneal allografts. Limbal LCs can be induced to migrate centripetally into the central cornea by instilling 1.0 μm sterile latex beads into shallow incisions in the corneal epithelium (6). Unlike untreated corneal grafts, latex bead-treated corneal grafts become infiltrated with donor-derived LCs and induce robust delayed type hypersensitivity (DTH) and cytotoxic T lymphocyte (CTL) responses to donor alloantigens (14). Moreover, the incidence of rejection doubles if donor LCs are present in the corneal allograft at the time of transplantation (14,15). However, the immune privilege of such corneal allografts can be restored if donor LCs are removed by either ultraviolet (UV) irradiation or treatment with hyperbaric oxygen (15).

The time-honored explanation for the immune privilege of corneal allografts is based on the assumption that the avascular corneal graft bed sequesters the corneal allograft from the peripheral immune apparatus, thereby preventing the emigration of donor alloantigens to peripheral lymphoid tissues and thwarting the immigration of host immune effector elements into the allograft. The previously mentioned findings indicate that any privilege provided by an avascular graft bed is lost if donor LCs are present in the corneal allograft.

The notion that "passenger cells" are important barriers for successful allograft survival was articulated almost a half-century ago (16) and has fallen in and out favor over the past 30 years. There is little doubt that passenger cells expressing donor alloantigens are capable of migrating from the graft and directly activating T cells. One of the properties of MHC antigens is their capacity to directly activate T cells without the participation of APCs. The so-called direct pathway of alloactivation is approximately 100 times more effective than activation by the indirect pathway in which host APCs reprocess donor alloantigens (17). The expression of MHC antigens and MHC-peptide complexes is 10 to 100 times higher on dendritic cells than other cells, and a single dendritic cell can activate up to 3000 T cells (18,19). On the surface one would predict that the MHC antigens expressed on passenger LCs arouse the greatest alloimmune responses and pose the greatest risk for corneal allograft survival. However, results from both rat and mouse models of penetrating keratoplasty indicate that donor LCs elicit strong alloimmune responses to donor minor histocompatibility antigens, which culminates in a two-fold increase in the incidence of corneal allograft rejection (15).

Although MHC antigens directly stimulate T cells, it is the minor histocompatibility antigens carried by the passenger LCs that pose the greatest risk to corneal allograft survival (Table 2).

HOST APCs AND THE INDIRECT PATHWAY OF ALLOIMMUNIZATION

Host-derived APCs also affect the fate of corneal allografts by processing donor histocompatibility antigens via the indirect pathway (17,20). Although the indirect pathway is less efficient than the direct pathway of alloactivation, it appears to play an important role in stimulating corneal allograft rejection (21,22). Four observations support the conclusion that host APCs and the indirect pathway of alloantigen presentation are important in the induction of corneal allograft rejection: (*i*) corneal grafts that confront the host with only minor histocompatibility antigens undergo immune rejection, even though these alloantigens are incapable of directly stimulating alloimmunity (23,24); (*ii*) removal of infiltrating donor LCs by either UV irradiation or hyperbaric oxygen treatment does not prevent the rejection of MHC-matched corneal allografts (15); (*iii*) depletion of conjunctival macrophages with a macrophagicidal drug (clodronate) prevents corneal graft rejection in rats (25); and (*iv*) inhibition of host LC migration from the graft bed into the corneal allograft significantly reduces corneal graft rejection (26).

APCs AS THERAPEUTIC TARGETS FOR PREVENTING CORNEAL ALLOGRAFT REJECTION

The importance of donor and host APCs in eliciting corneal graft rejection is undeniable. Studies in both mouse and rat models of penetrating keratoplasty indicate that the presence of donor-derived LCs dramatically increases the risk for alloimmunization and immune rejection (14,15,23).

Table 2 Effect of Donor LCs on Orthotopic Corneal Graft Rejection in Rodents

	% Graft rejection[a]	
Histoincompatibility	Normal corneal allograft	LC^+ corneal allograft
MHC + minor histocompatibility antigens	50%	>90%
Minor histocompatibility antigens only	40%	80%
Class I MHC only	18%	20%
Class II MHC only	<10%	<10%

[a]Results are summarized from data in Refs. 2–5.

Abbreviations: LC, langerhans cell; MHC, major histocompatibility complex.

Purging Donor APCs as a Means of Reducing the Immunogenicity of Corneal Allografts

UV irradiation and hyperbaric oxygen treatment have been successfully used to purge donor LCs from corneal allografts and reduce the incidence of immune rejection (15,27). UV irradiation is known to cause a sharp reduction in the numbers of LCs in the skin and impair their antigen-presenting function (28,29). UV treatment appears to have the same effect on corneal LCs, as UV treatment of LC-containing corneal allografts results in a steep reduction in the allospecific CTL and DTH responses to heterotopic and orthotopic corneal allografts and a commensurate reduction in graft rejection (30–32). Hyperbaric oxygen is also toxic to LCs and is effective in purging corneal allografts of infiltrating donor-derived LCs and reducing graft rejection (32). However, neither UV irradiation nor hyperbaric oxygen treatment completely prevents corneal graft rejection; both treatments appear to reduce corneal graft immunogenicity by inactivating or purging passenger LCs (14,15). That is, a significant number of UV- or oxygen-treated grafts undergo immune rejection, presumably because alloantigen presentation can proceed unimpaired via the indirect pathway. In the final analysis, using UV irradiation or hyperbaric oxygen treatment to purge corneal grafts of donor LCs may be unnecessary, as human corneal buttons held for three days in corneal storage medium have sharply reduced numbers of LCs (33). Moreover, there is evidence that corneal storage medium not only affects the viability of donor LCs but may also reduce the cornea's immunogenicity. Mouse corneal buttons briefly maintained in corneal storage medium are less immunogenic and fail to induce either CTL or DTH responses to donor alloantigens (34).

Targeting Host APCs as a Means of Preventing Corneal Graft Rejection

It is becoming increasingly clear that the indirect pathway of alloactivation plays a pivotal role in the induction of corneal graft rejection. For the indirect pathway to be activated, host APCs must be capable of capturing graft alloantigens and delivering them to a regional lymphoid tissue. Using a rat model of penetrating keratoplasty, Ross et al. demonstrated that host LCs migrated from the limbus into the body of the orthotopic corneal graft (23). Within 24 hours of penetrating keratoplasty, waves of host LCs could be observed at the interface of the graft and graft bed. LCs migrated centripetally and penetrated the central regions of the corneal allografts peaking at 3–4 days and persisting for over two weeks. Interleukin-1 (IL-1) is one of the first cytokines shown to induce centripetal migration of LCs into the central corneal epithelium (6). With this in mind, Dana et al. examined the effect of IL-1 receptor antagonist (IL-1RA) on LCs migration into orthotopic corneal allografts and found that topical application of IL-1RA resulted in a three-fold reduction in LC migration into corneal allografts (26) and a 50% reduction in graft rejection (35).

In addition to LCs, the tissues juxtaposed to the corneal graft bed also contain macrophages (36), which can function as APCs for the induction of alloimmune responses. Conjunctival macrophages can be effectively depleted by the injection of liposomes containing the macrophagicidal drug, dichloromethylene diphosphonate (clodronate) (25). Corneal allografts transplanted to clodronate-treated graft beds fail to induce either CTL or DTH responses to donor alloantigens and do not undergo immune rejection (37). These results represent a confounding paradox for understanding the role of LCs and macrophages in the induction of corneal alloimmunity and corneal graft rejection. Clodronate appears to be toxic only to macrophages and does not adversely affect dendritic cells (38) or other leukocyte populations (39). Therefore, the capacity of sub-conjunctival injection of clodronate-encapsulated liposomes to completely prevent corneal allograft rejection suggests that either: (*i*) LCs are not capable of inducing corneal allograft rejection; (*ii*) clodronate is in fact toxic to LC; or (*iii*) LCs require some heretofore unidentified interaction with macrophages in order to induce alloimmunity and corneal graft rejection. The weight of evidence to date indicates that LCs are crucial for the induction of corneal allograft rejection. The latter two explanations for the clodronate effect on corneal graft rejection warrant serious investigation. The capacity of clodronate-encapsulated liposomes to completely prevent corneal allograft rejection in the rat (25) and mouse (40) suggests that this therapeutic strategy holds great promise.

CONCLUSIONS

The use of topical corticosteroids has revolutionized corneal transplantation and remains the mainstay of the corneal transplant surgeon. However, immunosuppressive drugs, such as corticosteroids, have limited efficacy in high-risk hosts who have rejected previous corneal transplants or who have vascularized graft beds. Targeting host and donor-derived APCs is particularly attractive in these settings. Unlike corticosteroids, which carry considerable toxic side effects, manipulation of APCs in the graft and graft bed has little to no toxicity and has the added advantage of specifically handicapping the host's immune response to donor histocompatibility antigens. However, further work is needed to distinguish the roles of LCs and macrophages in eliciting alloimmune responses to corneal alloantigens. Once armed with this information, it might be possible to not only prevent the induction of unwanted alloimmune responses, but also to alter host APCs in a manner that leads to immune tolerance to donor alloantigens.

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14

The Role of Corneal Antigen-Presenting Cells in Herpes Simplex Keratitis

Robert L. Hendricks
Department of Ophthalmology, Immunology, and Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

HERPES SIMPLEX KERATITIS

Herpes simplex virus-introduced stromal keratitis (HSK) is a frequent infectious cause of corneal blindness. We have employed a mouse model to study this disease. Following corneal infection with the RE strain of HSV-1, 100% of mice, regardless of genetic background, develop epithelial lesions. In immunologically normal mice, these lesions typically heal within 2–4 days of their appearance and are not associated with permanent loss of vision. HSK subsequently develops in 60% to 80% of A/J mice and 80% to 100% of Balb/c mice. In this model, HSK represents a chronic inflammation that typically progresses from a mild corneal opacity (scored 1+), through a moderate opacity with emerging neovascularization (scored 2+), to severe opacity that obscures the view of the iris with ingrowth of blood vessels to the central cornea (scored 3+). If allowed to do so, inflammation in many of the corneas will progress to perforation (scored 4+). $CD4^+$ T cells that infiltrate the cornea produce the Th1 cytokine IFN-γ [that regulates neutrophil (PMN) extravasation from blood vessels in the limbus], and IL-2 (that regulates IFN-γ production, PMN chemotaxis, and PMN activation) (1–5). Mice that develop HSK and those whose corneas remain uninflamed after HSV-1 infection both develop a predominantly Th1 cytokine pattern in the lymph nodes of similar magnitude (6). Thus, a critical issue in

understanding the pathogenesis of HSK is defining how Th1 cytokine production is regulated within the HSV-1 infected cornea.

ROLE OF CORNEAL APCs IN HSK

Recent studies from our laboratory (7) demonstrated the presence of bone marrow-derived cells within the normal mouse cornea. Numerous $CD45^+$ cells with pleomorphic and dendriform morphology were found within the pericentral and central region of the corneal stroma (200–300 cells/mm^2). Dual-color immunostaining demonstrated that 100% of the $CD45^+$ cells co-expressed CD11b and 50% co-expressed F4/80. Approximately 30% of the total cells and 50% of the $F4/80^+$ cells co-expressed major histocompatibility complex (MHC) class II antigens. Very small to negligible numbers of cells expressed markers of dendritic cells (CD11c) or granulocytes (Ly6G). Markers for T-cells and NK cells were absent from the corneal stroma, indicating that all the cells identified in the stroma were of the myeloid lineage. Studies by M. Reza Dana (see Chapter 12) also described a network of macrophage-like bone marrow-derived cells in the normal mouse cornea, but their findings conflicted with ours in that they also found cells that expressed the dendritic cell marker CD11c (8,9). Both laboratories found that the majority of bone marrow-derived cells lacked expression of MHC class II, raising interesting questions about the potential role of these corneal cells as immunogenic or tolerogenic APCs.

Although the function of endogenous APCs in the normal cornea remains unresolved, there is no question that trauma to the central cornea induces infiltration of Langerhans cells (LCs), a population of DCs that reside in the limbal region of the normal cornea. Such infiltration was shown to occur following HSV-1 infection of the mouse cornea (10). Moreover, depletion of LCs prior to HSV-1 corneal infection inhibited the development of HSK, and also reduced the DTH response to HSV-1 in the ear pinna. These findings indicated that corneal DCs are important for the induction of the $CD4^+$ T-cell response to HSV-1 in the draining lymph nodes, but it was not clear if DCs that infiltrated the cornea after infection were also required to re-stimulate the effector phase of the $CD4^+$ T-cell response within the cornea. This issue was resolved by depleting DCs from only one cornea of a mouse followed by bilateral HSV-1 corneal infection. This treatment inhibited HSK in the DC-depleted cornea, but not in the companion cornea, demonstrating a role for DCs in the effector phase of the $CD4^+$ T-cell response that regulates HSK.

DCs reside in tissues in an immature state characterized by high endocytic capability, but low expression of MHC and costimulatory molecules required to activate naïve T cells. Under the influence of a variety of inflammatory cytokines, DCs mature into cells that have low endocytic capability, but express high levels of MHC and costimulatory molecules such as B7 and CD40. However, data was presented demonstrating that HSV-1 infection inhibited the lipopolysaccharide (LPS)-induced maturation of DCs as indicated by reduced expression

of B7-1, B7-2, and MHC class II (unpublished data). DC maturation was not inhibited following infection with ultraviolet light inactivated HSV-1, which expresses few, if any, viral genes. Thus, viral gene expression appears to be required for inhibition of DC maturation (unpublished data). Our laboratory also demonstrated that the B7-1/CD28 costimulatory interaction is required for the effector phase of the $CD4^+$ T-cell response that regulates HSK (6). We also demonstrated that depletion of T cells from mice for 30 days starting at the time of HSV-1 corneal infection prevented HSK development (11). However, when T-cell depletion was discontinued, the mice developed HSK. Together these findings raised an interesting paradox: How can DCs effectively induce a T-cell response to HSV-1 if (*i*) their maturation is inhibited by the virus and (*ii*) HSK can initiate more than 30 days after HSV-1 is eliminated from the cornea?

It appears that the majority of DCs that enter the HSV-1 infected cornea do so 7–10 days after the replicating virus is eliminated from the cornea (6). If these cells are involved in presenting viral antigens to infiltrating $CD4^+$ T cells, where do they acquire the antigens? We propose two possible sources of viral antigens. One source could be the apoptotic or necrotic cells that are present in the cornea following elimination of the replicating virus. This possibility would suggest that cross-presentation of antigens that are acquired by phagocytosis rather than direct presentation of viral antigens by infected DCs is primarily responsible for activation of $CD4^+$ T cells in the cornea. This route of antigen acquisition would negate the effect of viral gene expression on DC maturation. This possibility is supported by the recent findings of Carbone et al. (12,13) that the DC population responsible for presenting HSV-1 antigens to CTL in the lymph nodes is not the population that is present at the site of infection. Thus, cross-presentation might be a general means used by DCs to avoid the inhibitory effect of certain viral gene products on DC maturation.

Although the phagocytosis of apoptotic cells and cell debris containing viral antigens might account for the development of HSK 7–10 days after corneal infection, it would not seem to account for the development of HSK more than 30 days after infection. Evidence was presented suggesting that some HSV-1 lytic gene products (notably glycoprotein B) are produced in latently infected sensory neurons, and recognized by $CD8^+$ T cells within the ganglion (14). A proposal was made that viral glycoproteins that are produced in latently infected sensory neurons might be transported down nerve axons to the cornea, where they can be acquired and cross-presented to HSV-1 specific $CD4^+$ T cells. This could explain why certain individuals develop recurrent bouts of HSK in the apparent absence of infectious virus. This hypothesis is currently being investigated.

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15

Antigen-Presenting Cells and the Eye: Bacterial and Parasitic Infections

Linda D. Hazlett
Department of Anatomy and Cell Biology, Wayne State University, Detroit, Michigan, U.S.A.

INTRODUCTION

Dendritic cells, one of the most potent of the antigen-presenting cells (APCs), have a unique ability to induce primary immune responses against microbial infection (1). They also play a major role in acquired immune responses (2). Immature dendritic cells capture antigen and then migrate to regional lymph nodes where they develop into mature cells. Mature APC are then able to activate antigen-specific naïve lymphocytes. Immature dendritic cells express low levels of major histocompatibility complex (MHC) class II, CD83, and costimulatory molecules such as CD80 (B7-1), CD86 (B7-2), and CD40; mature dendritic cells are characterized by high expression levels of these marker molecules (3,4). These cells may be thought of as architects of immunity (5). Dendritic cells not only present antigen extremely efficiently, determining the magnitude of the immune response, but also influence the quality of the response, contribute to deletional tolerance, promote cross-priming, and may be important in downregulation of effector responses and the maintenance of memory (6).

There are two categories of dendritic cells based on their abilities to mount a Th1 or Th2 response (7,8). Dendritic cells (1) produce IL-12, which stimulates naïve $CD4^+$ T cells to develop into mature Th1 T cells, whereas dendritic cells (2) skew naïve cell differentiation to the Th2 pathway (9). Dendritic cells can also be separated into distinct subsets according to their myeloid or lymphoid origin. In mice, the myeloid-related dendritic cells are $CD11c^+$, $CD11b^+$, $CD8a^-$, and

DEC205^{-}, and the lymphoid-related dendritic cells are CD11c^{+}, CD11b^{-} CD8a^{+}, and DEC 205^{+} in phenotype (10,11). The normal cornea, once thought to be an immune privileged site and to lack these kinds of cells, has been shown recently to contain them at different stages of maturity, dictated by their residence site within the tissue (12). This new information is obviously important to all studies examining immune mechanisms at this site.

In infectious disease, microbial molecules are capable of activating immature dendritic cells to become mature. The latter are characterized by cytokine production, upregulation of costimulatory molecules, and increased ability to activate T cells through Toll-like receptors (TLRs) (13,14). The latter, an important receptor family comprised of functionally distinct members, is activated by microbial infection (15).

LANGERHANS CELLS

Langerhans cells are APCs that localize in skin (16) and in various mucosal sites, including the eye (17,18). These cells constitutively express MHC class II antigen and until recently (12) were thought to be normally absent from the central cornea (19). Many diverse stimuli or irritants, including infectious (20,21), non-infectious (22,23), and, of the latter, experimental extended wear contact lens usage (24), initiate a centripetal migration of these cells from the conjunctival epithelium into the adjacent cornea. Langerhans cells in the conjunctiva are immature cells that exhibit limited antigen-presenting function, but cytokines produced by cells of the cornea (25) likely stimulate both the migration and maturation of these cells (26). Our experimental studies of Langerhans cell migration into the central cornea following extended wear contact lens usage in a rabbit model suggested that in human patients, use of extended wear contact lenses could induce the cells into the cornea. Furthermore, their presence there may account for the routinely described rapid host response to the bacterial pathogen, *Pseudomonas aeruginosa*, classically associated with infection in lens-wearing patients. These infections often result in corneal scarring, reduced visual acuity, and/or perforation, despite vigorous antibiotic treatment. Our data suggest that the presence of Langerhans cells in the cornea could prime the lens-wearing eye to a more rapid initiation of antigen processing (22,27) and enhanced immune responsiveness. In contrast, the presence of Langerhans cells in corneas exposed experimentally to Acanthamoeba-laden lenses resulted in augmented antigen presentation and proved to be beneficial in the prevention of development of keratitis (28). In stark contrast to the potential benefit of the cells in cornea in such cases of parasitic disease is their deleterious effect in immunopathological diseases involving antigen presentation to T cells, such as occurs in experimental herpetic infections. In the latter, the end result is eradication of the pathogen, but concomitantly, host tissue is destroyed and loss of vision occurs.

To directly test the consequences of Langerhans cells in the cornea before infection with *P. aeruginosa*, the cells were induced centripetally from the

conjunctiva by topical sterile bead application onto the lightly scarified cornea (29) of susceptible (cornea perforates) C57BL/6 (B6) and resistant (cornea heals) BALB/c mice (30). We detected no difference in disease response in the bead- versus sham-treated B6 mice after infection; however, significant differences that eventually led to corneal perforation were observed in BALB/c mice. These included an increased number of ADPase (31) positively stained Langerhans cells in the central cornea of bead- versus sham-treated mice after infection and, more importantly, a significantly greater number of Langerhans cells in the cornea of the bead-treated mice were found to express positive immunostaining for the costimulatory molecule B7-1. Expression of this and other maturation markers has been shown to enhance the capacity of the cells to present antigen to $CD4^+$ T cells (32). Remarkably, the presence of Langerhans cells in BALB/c cornea before infection also was associated with the localization of activated $CD4^+$ T cells in the cornea that were detectable at 5 days after infection. No T cells are ever detected in the infected BALB/c mouse cornea that heals, but these cells are routinely detected in the infected cornea of B6 mice whose cornea perforates between 5–7 days after infection. The cell infiltrate in the stroma of bead- versus sham-treated cornea also differed. Rather than the routinely expected polymorphonuclear neutrophilic (neutrophil) infiltrate, the stroma contained numerous large mononuclear-type cells that were confirmed as macrophages using transmission electron microscopy and acid phosphatase staining (30).

Systemically, delayed type hypersensitivity (DTH) as well and mRNA expression levels of IFN-γ were increased in both cornea and draining cervical lymph nodes in the bead-treated mice. In contrast, levels of IL-4 were significantly higher in the cornea and cervical lymph nodes of sham- versus bead-treated mice. We hypothesize that in BALB/c, IL-4 may participate with other cytokines (such as IL-10) (33) to mediate resistance due to the ability of the former cytokine to stimulate phagocytosis of neutrophils or potentiate neutrophil degranulation and respiratory burst (34), hastening bacterial clearance and downregulating the persistence of neutrophils in the cornea (35,36). In B6 mice, however, exogenous injection of the cytokine (recombinant protein) failed to completely rescue the susceptible phenotype (37).

Costimulation

The role of Langerhans cells and the B7/CD28 costimulatory pathway in *P. aeruginosa*-infected cornea and the contribution of costimulatory signaling by this pathway to disease pathology also have been studied (38). Langerhans cells were more numerous in the infected cornea of B6 versus BALB/c mice at various times after infection. In addition, mature, B7 positive-stained Langerhans cells in the cornea and *Pseudomonas* antigen-associated cells in draining cervical lymph nodes were also increased post infection in susceptible versus resistant mice. When B6 mice were treated both sub-conjunctivally and systemically with neutralizing B7 (B7-1/B7-2) monoclonal antibodies, disease severity was reduced

and the number of B7-positive cells as well as the recruitment and activation of CD4+ T cells in the cornea were significantly reduced. Expression levels of mRNA for IFN-γ also were decreased significantly in the cornea and in draining cervical lymph nodes of antibody-treated mice. When B6 mice endogenously lacking CD28 were tested, they also exhibited a less severe disease response (no perforation) when compared with wild type mice. In addition, knockout mice had a significantly lower DTH response to heat-killed *Pseudomonas* antigen, supporting a critical role for B7/CD28 costimulation in susceptibility to *P. aeruginosa* ocular infection. In other systems, the capacity of B7 to deliver a costimulatory signal to T cells by binding to CD28 is well documented both in vitro and in vivo (39). Systemic treatment with monoclonal antibody to B7 can reduce the severity of T cell-mediated inflammatory processes such as autoimmune encephalomyelitis (40) and experimental lupus (39). Reduction of cytokine and chemokine levels also has been reported using anti-B7 monoclonal antibodies. For example, production of MIP-1α induced following TCR-mediated T-cell activation was inhibited with anti-B7 monoclonal antibodies, indicating that full production of this C-C type chemokine depended on interaction with B7 ligand through interaction with CD28 (41). These interactions also appear consistent with the role of MIP-1α in T-cell chemotaxis in the susceptibility response as described previously (36).

We next addressed the questions of whether or not lymph nodes (cervical) were required as the site of antigen presentation by Langerhans cells to naïve T cells and whether the subsequent T-cell response in cornea was antigen-specific or nonspecific. Studies focused on these issues were initiated (42) and involved surgical removal of the draining cervical lymph nodes in B6 mice followed by challenge with *P. aeruginosa* and subsequent immunostaining for CD4+ T cells in cornea. We found that whether or not lymph nodes were present (sham surgery) or surgically removed, CD4+ T cells that were activated (CD25+) remained detectable in the cornea. This is not altogether surprising, as T cells are detected early in cornea after infection (3 days) and are activated at 5 days after infection. It is probable, but not yet proven, that the infected cornea provides signals that regulate chemotaxis of T cells in the conjunctiva to migrate into the cornea where they are then non-specifically activated. Experiments to test the latter, using mice with selective ova-antigen-specific T-cell responsivity, are underway.

DENDRITIC CELLS AND NEUROPEPTIDES

The cornea is one of the most densely innervated tissues in the body. It is richly supplied by both sensory and autonomic nerve fibers. Corneal innervation has become of increased importance in recent years due to the observation that corneal nerves are routinely injured following certain corneal diseases as well as refractive surgeries (43). In the epithelium and stroma, nerve fibers containing sensory neuropeptides including, but not limited to, substance P (SP), calcitonin gene-related peptide (CGRP), and vasoactive intestinal peptide (VIP) have been

described for human (43) and murine cornea (Hazlett, unpublished data). These and other neuropeptides have been identified as potent mediators of inflammatory and immunological reactions involving leukocytes, including dendritic cells. These cells can be found in the airway epithelium, for example, and unlike other immune cells that rapidly transit into the airways, dendritic cells remain within the epithelium during an acute inflammatory response. In this regard, investigating the effects of these nervous system-derived mediators on the migratory behavior of human peripheral blood derived mononuclear cells used to generate dendritic cells, revealed contrasting responses in migration of the cells using Boyden chamber assays. In immature cells, secretin, secretoneurin, VIP, and CGRP induced dendritic cell chemotaxis in vitro comparable to RANTES (the positive control). SP and CCL19 stimulated immature cell chemotaxis only slightly. Responses of the dendritic cells to neuropeptides depended on the maturation state of the cell. Peripheral neuropeptides directly attracted immature dendritic cells to peripheral nerve fibers where high concentrations of the peptides arrested the mature cells. This behavior was accompanied by changes in signal transduction pathways of neuropeptide receptors in both immature and mature cells (44). It was hypothesized that one function of sensory nerves is to fasten dendritic cells at sites of inflammation. Although eye-derived Langerhans cells have not yet been tested similarly, we have tested a murine Langerhans cell line (XS52) derived from BALB/c skin and the data are shown below in Figure 1. Immature murine Langerhans cells exhibited a different chemotactic response pattern than derived human cells in that RANTES (positive control)=SP>SN>VIP>media control.

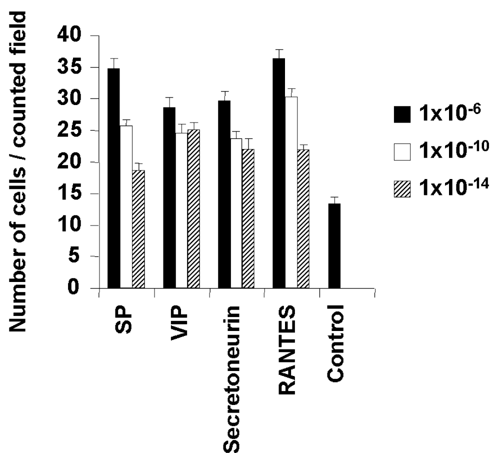

Figure 1 Murine dendritic (Langerhans) cells were tested for chemotaxis in a Boyden chamber assay. Cells from six fields per agent per concentration were counted and the data are significant (P=0.0001 for all when compared with media control). *Abbreviations*: SP, substance P; VIP, vasoactive intestinal peptide. *Source*: From Ref. 44.

Chemotaxis was dose dependent, as described by Dunzendorfer et al. (44) for human-derived dendritic cells. Although further testing will be required in this system as well as with eye-derived Langerhans cells, the disparate data may reflect species differences, and/or the source of the cell being tested.

DENDRITIC CELLS AND TOLL-LIKE RECEPTORS

Activation of innate immunity through pattern recognition receptors for ligands derived from evolutionary distant pathogens provides essential signals for initiation of the adaptive immune response (45,46). Microbial infection activates the TLR signaling cascade (47), resulting in expression of various pro-inflammatory cytokines, chemokines, and large quantities of small antimicrobial peptides such as the defensins (48,49). Recent evidence suggests that murine β-defensin-2 acts directly on immature dendritic cells as an endogenous ligand for TLR-4, inducing upregulation of costimulatory molecules and dendritic cell maturation that in turn triggers a robust type 1 polarized adaptive immune response (50). Thus, TLRs are critical in both the innate immune response, functioning as recognition receptors for pathogen-specific molecules (51) and in the subsequent adaptive immune response leading to resolution (or worsening) of disease.

Despite the importance of these molecules, the role of TLRs in *P. aeruginosa* keratitis is not well understood (52). Our gene array data showed that the expression of TLRs and related molecules including CD14, soluble IL-1 receptor antagonist, TLR-6, and IL-18R-accessory-protein are significantly elevated in susceptible (cornea perforates) versus resistant (cornea heals) mice (53), suggesting an important immunomodulatory role for these molecules that may influence early as well as later events that occur in the disease response resulting in the susceptible versus resistance phenotype.

Microarray analysis combined with quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) provided a comprehensive view of the genetic events characterizing the initial development of pathology in *P. aeruginosa* keratitis (53). Results provide insight regarding unappreciated mechanisms of pathogenesis and possible unique targets for therapy of this destructive inflammatory disease. These data regarding TLR and CD14 in the cornea of infected mice are complemented by work in the Ansel laboratory (54), who demonstrated for the first time that human corneal cells (epithelial, stromal, and endothelial) are capable of expressing the functional (lipopolysaccharide) LPS receptor complex proteins CD14 and TLR-4. mRNA expression was determined by RT-PCR, and Northern blot and cell surface expression of these proteins was detected by flow cytometry. The cellular cytokine and chemokine expression was measured by enzyme-linked immunosorbent assay (ELISA). All three cell types of the human cornea expressed CD14 mRNA and cell surface CD14. LPS binding to CD14 resulted in a rapid intracellular calcium response and the secretion of multiple pro-inflammatory cytokines and chemokines. CD14 mRNA expression in corneal epithelial cells was upregulated by LPS. In addition to CD14, corneal epithelial

cells expressed the functional LPS receptor-signaling protein TLR-4, which was also augmented by LPS. Nonetheless, these data are not supported by others.

Other investigators (52) identified specific mediators of LPS-induced keratitis in vivo. BALB/c, C3H/HeN, and C3H/HeJ (LPS hyporesponsive) mice received corneal abrasion to the epithelium and LPS from *P. aeruginosa* (10μg/1μl) topically. Stromal thickness and haze were measured by in vivo scanning confocal microscopy and neutrophil recruitment determined by immunochemistry. LPS from *P. aeruginosa* produced a significant increase in stromal thickness and haze compared with untreated control corneas, and disease severity coincided with neutrophil infiltration into the stroma. Systemic depletion of neutrophils completely abrogated LPS-induced increases in stromal thickness and haze. Expression of platelet endothelial cell adhesion molecule (PECAM-1) on vascular endothelium and production of MIP-2 in the corneal stroma were also significantly elevated after LPS exposure and antibody blockade inhibited neutrophil recruitment to the cornea and abrogated LPS-induced increases in stromal thickness and haze. In C3H/HeJ mice that are LPS hyporesponsive, PECAM-1 and MIP-2 were not upregulated after LPS exposure and endotoxin-induced keratitis did not develop in these mice. The findings demonstrated that endotoxin-induced keratitis is regulated by TLR-4 dependent expression of PECAM-1 and MIP-2 that are essential for recruitment of neutrophils to the corneal site and for development of LPS-induced stromal disease. Clearly, further work is needed in this area to fully elucidate the significance of these important molecules both in sterile and infectious keratitis.

DENDRITIC CELLS AND PARASITIC INFECTION

There is increased recognition that dendritic cells are an important source of the IL-12 required to initiate protective immunity to parasites such as *Leishmania* and *Toxoplasma.* In addition, there appear to be differences in the dendritic cell activation pathways utilized by these two intracellular protozoa, which may also differ from the pathways used by bacteria. *Toxoplasma* and *Leishmania* prime dendritic cells, allowing responsiveness to CD40 ligation, which promotes IL-12 production (55–57). In the case of *Toxoplasma*, this priming may involve signaling through the chemokine receptor, CCR5 (58). Treatment of dendritic cells with pertussis toxin (that blocks G-protein signaling) blocks almost all IL-12 production. In contrast, in *Leishmania* infection (*L. major*, in BALB/c mice), pertussis toxin enhances IL-12 production (59). Clearly, pathways leading to dendritic cell activation after infection are just beginning to be explored and numerous questions remain. Some of the more important include: defining the pathogen-derived molecules, and the dendritic cell receptors that are responsible for activating dendritic cells; defining the role of dendritic cells in the maintenance of immunity; and assessing whether definable subsets of dendritic cells are responsible for priming cells to become Th1 or Th2 cells. There are no published studies that have similarly investigated the role of these cells in parasitic infections of the eye.

DENDRITIC CELLS AND PROTECTION AGAINST INFECTION

The interaction of *P. aeruginosa* with dendritic cells was evaluated and the use of dendritic cells pulsed with the bacteria to induce protection against fatal pulmonary infection with *P. aeruginosa* tested. Bone marrow-derived dendritic cells interacted with and were activated by the bacteria in vitro and dendritic cells pulsed with the bacteria and administered to syngeneic mice lead to induction of $CD4^+$ T-cell proliferation and prolonged survival after a lethal intrapulmonary challenge. It also was determined the presence of $CD4^+$ T cells was required for beneficial outcome (60).

In another study (61), dendritic cells genetically engineered with a recombinant adenovirus vector to express CD40 ligand were tested using several different strains of *P. aeruginosa* to determine whether such a strategy is applicable to enhancing clinically relevant pathogen-specific immunity. Immunization of mice with dendritic cells modified with CD40 ligand and pulsed with heat-killed *P. aeruginosa* (isolated from an individual with cystic fibrosis) survived a lethal respiratory challenge. The protected mice generated high levels of serum isotype-switched antibodies directed against the infecting bacterial strain without non-specific elevation of total serum immunoglobulin levels. The CD40 ligand genetically engineered dendritic cells pulsed with seven of eight different *P. aeruginosa* strains afforded significant but variable cross-protection following similar challenge with the isolated bacterial strain used in the initial test. In contrast, $CD4^+$ T cells were not found to be required. Although yet in early stages, these studies may prove useful in vaccine development against not only *P. aeruginosa* infections, but other microbial diseases as well (61).

Similar types of studies have evaluated dendritic cell-based immunotherapy for treatment of established murine visceral leishmaniasis. Repeated injection of *L. donovani*-pulsed dendritic cells failed to completely clear the parasite from liver and spleen. However, conventional anti-leishmanial chemotherapy (sodium antimony gluconate) along with injections of parasite-pulsed dendritic cells resulted in complete clearance of parasites from both of these organs (62).

FUTURE DIRECTIONS

Experimental models of bacterial infection with *P. aeruginosa* have provided important insights into the mechanisms underlying ocular inflammation. However, our understanding and knowledge of the precise mechanisms operative in human cases of keratitis (sterile and infectious) is much more limited. Studies will be needed to elucidate the mechanisms of disease induced by bacterial as well as host factors and to define with precision the cascade of events occurring at the onset of inflammation where the role of the neutrophil appears critical. It is intriguing to speculate that modulation and/or control of Langerhans cells may hold the key to regulation of inflammation within the cornea and ocular adnexa.

A clearer understanding of the interplay of effector and regulatory cells is also required within the eye. Although the study of TLRs is still in its infancy, with

complex questions remaining (63), the field has made rapid strides to explain many aspects of our ability to respond to pathogens. However, relatively little is known about this pathway in the eye, and questions regarding the agonist recognition systems and signaling pathways of these molecules remain unexplored. It is tempting to speculate that their exploitation may generate new therapies for inflammatory diseases. For example, reducing excess inflammation by downregulating TLR responses, together with conventional antibiotic therapy, is a likely feasible goal. It also remains speculative that RNA interference (post-transcriptional gene silencing) could hold promise, not only experimentally, but also in clinical modulation of eye disease, by the ability to silence a selected TLR signaling component(s).

Uncovering information about unconventional modulators of immune responsiveness such as the neuropeptides [e.g., the anti-inflammatory role of VIP (64–66)] and their regulation of T cells, as well as of cellular chemotaxis (e.g., dendritic cells) (44), is another avenue that holds promise for therapeutic intervention, particularly as the cornea is one of the most richly innervated mucosal tissues in the body (43). In this regard, increased reports of emergence of antibiotic-resistant bacterial strains provide further impetus to better understand the mechanisms of host–pathogen interaction and inflammatory events in the eye. It is expected that these studies will yield novel targets for more successful treatment of ocular inflammatory diseases.

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16

Skin Allergy Versus Ocular Allergy

Natalija Novak and Thomas Bieber
Department of Dermatology, University of Bonn, Bonn, Germany

SKIN ALLERGY

Atopic dermatitis (AD) is a chronic, inflammatory, allergic skin disease that results from complex interactions between genetic and environmental mechanisms (1). Together with allergic rhinitis, allergic asthma, and allergic rhinoconjuncitivitis, AD belongs to the so-called "atopic diathesis" (2,3). AD offers a wide clinical spectrum consisting of relapsing eczematous skin lesions with a typical predilection in the flexural folds of the body (4,5).

One characteristic feature of this disease is the reduced epidermal skin barrier, which results from xerosis and an enhanced transepidermal water loss combined with an altered lipid composition and pH changes to alkalinity (6).

Due to this impairment of the epidermal skin barrier, bacteria, allergens, and viruses can invade the epidermis in AD patients.

Another characteristic feature in the pathophysiologic puzzle of AD is a hyper-reactivity of epidermal effector cells, such as mast cells and basophils (7).

Allergens that invade the epidermis bind to allergen-specific IgE molecules on the surface of mast cells. This leads to the activation of the signal transduction cascade of these cells and the rapid release of pre-formed mediators such as histamine and leukotriene.

Since the discovery of IgE binding, CD1a-positive dendritic cells (DCs) in the epidermis of AD patients more than a decade ago, two distinct IgE-binding DC subpopulations that bear the high affinity receptor for IgE (FcεRI) on their

cellular surface have been identified: first, the classical Langerhans cells (LCs), which are CD1a and FcεRI positive and are characterized by their typical tennis racket shaped Birbeck granules; and secondly, another CD1a-positive DC cell population, which displays a high FcεRI surface expression—the so-called inflammatory dendritic epidermal cells (IDECs), which, in contrast to LCs, do not have any Birbeck granules. While LCs can be found also at non-lesional and healthy skin sites, IDECs are only present at inflammatory skin sites.

Allergens, which can invade the skin due to the reduced skin barrier in AD patients, are taken up by FcεRI-bearing LCs, internalized, and efficiently channeled into major histocompatibility complex (MHC) II compartments. After successful allergen processing, they are presented to allergen-specific T-cells, which leads to the allergic inflammation in the skin of these patients.

Furthermore, there is an intrinsic defect of keratinocytes in the skin of AD patients. Keratinocytes of AD patients produce enhanced amounts of pro-inflammatory cytokines and chemokines such as interleukin (IL)-8, tumor-necrosis factor (TNF)-α, IL-1β and granulocyte-macrophage-colony stimulating factor (GM-CSF) or soluble factors such as thymic-stromal lymphopoetin (TSLP).

In addition, there is high expression of Fas ligand (Fas-L) and the Fas antigen, keratinocyte–apoptosis, and an upregulation of the ICAM-1 expression of these cells (Fig. 1).

Recently, we showed that FcεRI activation of LCs leads to the release of chemotactic signals, such as interleukin (IL)-16, monocyte-chemoattractant protein (MCP)-1, macrophage-derived-chemokine (MDC), and thymus- and activation-regulated chemokine (TARC), which might contribute to the recruitment of inflammatory cells, such as IDEC, from the blood into the skin of AD.

Further on, there is an enhanced recruitment of eosinophils and T cells of the Th2 type, which produce high amounts of IL-4, IL-5, and IL-13 into the allergic-inflammation of the skin.

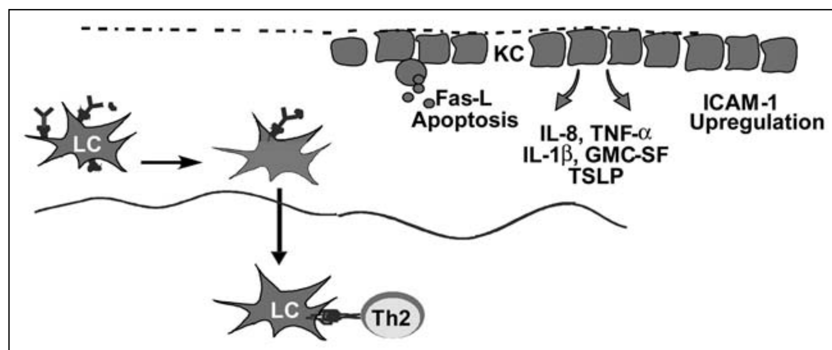

Figure 1 Allergen uptake by Langerhans cells (LCs) in the skin, and in the intrinsic defect of keratinocytes (KC).

The skin of AD patients is highly colonized with *Staphylococcus aureus* bacteria, which produce enterotoxins. These toxins act as so-called superantigens.

Under normal conditions, the epidermal compartment harbors highly efficient defense mechanisms of the innate immune systems against invading microbial components, which consist of the so-called defensins. Defensins, as implicated by their name, are capable to defend immediately and efficiently against invading microbial products.

Recently, it has been shown that the amount of beta defensins and cathelicidin is dramatically reduced in the skin of AD patients in comparison to patients with other inflammatory skin diseases such as psoriasis, and this might be the reason for the high frequency of bacterial superinfection in AD patients.

Another interesting component which contributes to the chronification of the skin lesions are the autoallergens. Autoallergens are intracellular proteins that can be released in consequence of mechanical damage such as scratching.

These autoantigens can activate mast cells, which release histamine. This may lead to an itch-scratch circle in these patients.

Further on, autoallergens can activate antigen-specific cells leading to the activation of autoreactive T cells and the amplification of the inflammatory immune response in the skin of these patients.

In view of these data, a picture emerges that allergens invading the skin of AD patients activate effector cells and FcεRI-bearing APCs. This induces the release of chemotactic signals and the recruitment of inflammatory cells such as IDECs into the skin lesions.

Together with the release of pro-inflammatory mediators by keratinocytes, the release of IL-12 and IL-18 by IDEC initiates the switch of the initial immune response of the Th2 type into an immune response of the Th1 type in which interferon-γ (IFN-γ) producing T cells predominate, which leads to the chronification of the skin lesions.

OCULAR ALLERGY

Comparing skin allergy, such as AD, and ocular allergy, such as allergic keratoconjunctivitis, several similarities can be found.

Elevated serum IgE levels and allergen-specific IgE can be detected in both skin allergy and ocular allergy. Most importantly, there is an overlap between these two groups of patients, since 15% to 40 % of AD patients have ocular involvement and suffer in addition to their skin lesions from atopic keratoconjunctivitis (8,9).

Impairment of the ocular surface epithelium over the conjunctiva and cornea is caused by a variety of factors, such as direct effects of eosinophil mediators, like eosinophilic cationic proteins (ECP) and major basic protein (MBP), and reduced IgA level and effects of exotoxins from *S. aureus* bacteria (Fig. 2) (8,9).

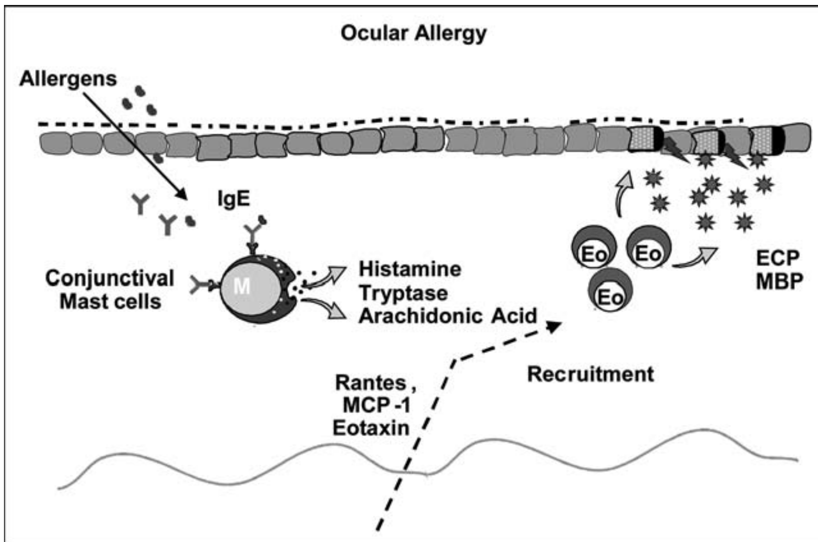

Figure 2 Allergen stimulation of conjunctival mast cells.

Therefore, aeroallergens such as ragweed and grass pollen or house dust mite allergens can invade into the eye.

These allergens acitvate mast cells, which are the earliest responder to allergen challenge, leading to the degranulation of pre-formed mediators such as histamine and tryptase and newly synthesized mediators such as arachidonic acid and leukotrienes. Mast cells also show an enhanced production of inflammatory mediators such as cytokines and chemokines.

This leads to a defect of conjunctival epithelial cells, which release the apoptotic factors Fas-L, upregulate ICAM-1 expression, and produce proinflammatory mediators such as IL-8, TNF-α, and GM-CSF (Fig. 3) (8–10).

In addition, an enhanced number of CD1a$^+$ DCs, which bear high amounts of IgE molecules on their cellular surface, can be detected after ocular allergen challenge in the conjunctiva of these patients.

CONCLUSION

Although there are numerous similarities between skin allergy and ocular allergy, several aspects such as the existence of inflammatory DC subtypes and the role of autoallergens or superantigens in allergic ocular diseases remain to be verified. Unraveling similarities and differences between skin allergy and ocular allergy would form the basis for related therapeutic strategies that might be useful for the treatment of both skin allergy and ocular allergy in the future.

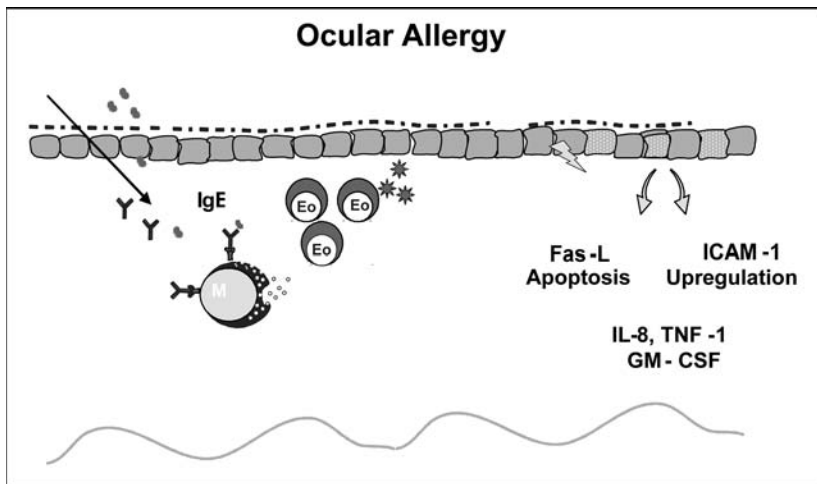

Figure 3 Damage of conjuctival epithelial cells.

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17

Antigen Presentation in the Eye: Uveitis

Janet Liversidge
Institute of Medical Sciences, University of Aberdeen, Foresterhill, U.K.

Patrick Tighe
Department of Immunology, Queens Medical Center, Nottingham, U.K.

Andrew Dick
Bristol Eye Hospital, Bristol, U.K.

John V. Forrester
Institute of Medical Sciences, University of Aberdeen, Foresterhill, U.K.

INTRODUCTION

The retina is considered a site of immune privilege, where immune responses must be controlled to protect vision. To achieve this, the need for immune surveillance to counteract infection must be balanced against the need to control potentially sight-damaging inflammation. A physiochemical barrier that restricts normal leukocyte diapedesis is provided by the blood–retina barrier, and soluble factors and immunomodulatory ligands on ocular-resident cells, designed to regulate immune responses locally and systemically, are well described and reviewed (1). More recently, it has become clear that ocular resident cells with antigen-presenting ability are programmed to suppress or tolerise infiltrating

T cells rather than activate them (2–5). Despite these multiple mechanisms, ocular inflammation involving the retina and uveal tract is not uncommon (5,6), indicating that the dynamic interactions between the cells and tissues of the eye and the immune system maintaining privilege can break down in response to infection or immune dysfunction. Posterior uveitis, the mononuclear cell inflammation of the retina and uveal tract that can result, is thought to represent an autoimmune response to retinal antigens (5,7,8), and in this review we examine some data supporting the notion that endogenous posterior uveitis (EPU) represents an autoimmune response. In addition, we will briefly consider the evidence for local priming of T cells to retinal autoantigens and evidence for local regulation of the response.

RETINAL AUTOANTIGENS

The retina is known to contain several potential autoantigens that have been implicated in the pathogenesis of EPU (9). The most abundant of these are S-antigen and interphotoreceptor retinoid binding protein (IRBP) (10). Both antigens are highly conserved between species, reflecting their importance in the process of vision, but both antigens contain epitopes that are also potent immunogens, inducing clinically and histologically similar $CD4^+$ T-cell-dependent autoimmune uveitis in a range of animal models (10–12).

Recent strategies for the treatment of $CD4^+$ T-cell-mediated autoimmune disease have focused on the possibility of altering the immune response by peptide therapy. This process involves blocking or modulation of the major histocompatibility complex (MHC)-peptide–T-cell receptor (TCR) recognition event that controls the immune response, either directly, or through manipulation of the immune system to re-establish a state of immunological tolerance to antigen. Both S-antigen and IRBP have been extensively studied to obtain information on immuno-dominant pathogenic or modulatory epitopes and the mechanisms by which they are recognized by the immune system (13,14). For this approach to succeed in vivo it is necessary to consider the effect of antigen processing on therapeutically administered antigen or peptide by host antigen-presenting cells (APCs). We have studied the proteases involved in antigen processing to predict which epitopes would be generated by APCs during normal processing. In vitro experiments had shown that S-antigen processing is independent of the ubiquitous endosomal protease cathepsin B, suggesting that S-antigen processing and peptide loading occurs outside the normal endo-lysosomal pathway (15). Further analysis of the role of cathepsins and acidic proteolysis in retinal antigen processing confirmed that processing of S-antigen is also independent of other cysteine proteinases and lysosomal enzymes in vivo. Different antigen processing pathways were found to be involved in the induction of experimental autoimmune uveoretinitis (EAU) by S-antigen and IRBP. IRBP was processed via conventional pathways requiring extensive proteolytic cleavage by cathepsins D and B. In contrast, our data suggested that an alternative pathway of antigen processing exists for S-antigen,

which is independent of processing within the normal endo-lysosomal pathway and that uveitogenic peptides of naturally processed S-antigen bind to MHC class II antigens either at the cell surface or within very early endosomes where cathepsin B is inactive (16). This diversity in MHC class II presentation, although highly unusual, is not unique. Other examples of aberrant processing have been detected in various disease states and it has been shown that epitopes bound to MHC class II in alternative sites are often associated with pathogen evasion of the immune system or induction of autoimmunity (17).

Why should soluble S-antigen taken up by APCs be processed via the alternative pathway, while soluble IRBP is processed through the conventional acidic proteolytic pathway? To answer this question, it was necessary to examine dendritic cell (DC) processing and antigen presentation function in induction of retinal autoimmunity.

The unique ability of DCs to activate naïve T cells and prime the immune response has been shown to depend on the differentiation status of the cell (18). DCs also control the nature of immune responses through the shaping of tolerance induction. The default role of tissue DCs is thought to be tolerance, thus tissue antigens acquired in normal tissues through uptake of apoptotic cells maintain peripheral tolerance, and inert particles in the gut and airway are "ignored" by DCs conditioned by the mucosal microenvironment (19). The key to induction of immunity is the triggering of specific pattern recognition receptors on DCs and other cells of the innate immune system by pathogens or by pro-inflammatory cytokines (20). Induction of autoimmunity to tissue antigens presented during a response to injury or infection should normally be prevented by central and peripheral tolerance mechanisms, but as retinal antigens are sequestered from normal immune surveillance, tolerance to them must be compromised. Furthermore, uveitogenic epitopes of S-antigen are known to contain microbial homologies (21,22) and a TNF-α homologous sequence (23). Crucially, the crystal structure of S-antigen shows that this epitope, GVXLXD, is located close to the amino terminal of the molecule well away from the rhodopsin-binding site, is exposed on the surface of the molecule, and is therefore available for recognition by ligands on other cells (24). We have also shown that the epitope is functional, and can elicit TNF-α-dependent responses in other cells (25). S-antigen was shown to drive maturation and differentiation of cultured DCs or choroidal DCs in tissue explants via the p55 TNF-α receptor. Choroidal DCs were stimulated to migrate and cultured DCs to accumulate surface MHC class II with a corresponding loss of acidic intracellular vesicles. DCs were also stimulated to express maturation dependent mRNA for the cytokines IL-1β and IL-12. S-antigen-pulsed DCs were also able to induce an immune response in vivo and to initiate Ig class switching. In contrast, IRBP-pulsed DCs appeared inert and had no priming effect in vivo (25). This data confirms that retinal antigens can have different effects upon the immune system and supports the hypothesis that exposure of the immune system to S-antigen in vivo, even in the absence of infection, could over-ride peripheral and ocular specific tolerance mechanisms and induce autoimmunity.

IMMUNE PROCESSES IN EYES OF UVEITIS PATIENTS

What evidence is there for an autoimmune response to retinal autoantigens in uveitis patients? Several studies have indicated that T-cell–mediated autoimmune processes play a major role (7,26), and that responses to S-antigen epitopes in particular may be involved (27–29). In addition, histopathological studies show that mononuclear cells pre-dominate in the inflammation, with granuloma formation consistent with a delayed type hypersensitivity response to antigens within the retina or uvea (30–32). Helper T cells and Th1 cytokines have been demonstrated in ocular fluids (8,32,33), but their relationship to the disease process is not well defined, as Th2 responses can also be damaging (34). Our studies have shown that vitreous cells are predominantly CD4 and CD8 T cells, and present in approximately equal numbers. These make up to 60% of the total infiltrate together with approximately 20% macrophages and 10% B cells (Fig. 1). Immunocytochemical analysis of cells showed that both T cells and macrophages also exhibited an activated phenotype. Large mononuclear cells strongly expressing MHC class II antigens were often associated with smaller lymphocytes, suggesting that cognate interactions between potential APCs and T cells were occurring within the vitreous and retina during uveitis.

Auto-aggressive T cells may use a restricted range of T-cell receptor (TCR) genes, so we compared the TCRβ chain repertoire of the activated ($CD25^+$) subset with total lymphocytes isolated from the peripheral blood of patients with clinically active disease, using data from healthy individuals as controls. Significantly elevated TCR Vβ1 usage within the $CD25^+$ T-cell population of patients was detected (35). To confirm peripheral bias was relevant to events in the disease site, vitrectomy samples were also examined. To detect local T-cell proliferation within the eye, T-cell clones present in the posterior chamber were examined using CDR3 spectra-typing. This method allows the polyclonality of an expressed TCR V gene family to be quantitatively determined on the basis of variable (V), diversity (D), and joining (J) (VDJ) gene segment lengths. Oligoclonality was observed in 3 out of 5 patients, but no evidence of TCR Vβ1 bias was detected in these samples. Figure 2 shows the results of one CDR3 spectra-typing experiment carried out to detect oligoclonality within each Vβ family within the peripheral blood, the vitreous, and from the sub-retinal space of a uveitis patient undergoing surgery. Polyclonality of each Vβ family within the circulation can be seen, with oligoclonality or even monoclonality evident within the vitreous and sub-retinal space for some families. In particular dominant monoclonality of Vβ6, Vβ12, Vβ15, Vβ17, and Vβ22 families in the sub-retinal space is evident, indicating epitope-specific proliferation is occurring within the retina. The number of Vβ families involved also suggests that the immune response is to more than one epitope in this region, and the oligoclonality of response in the vitreous indicates T-cell epitope spreading to other ocular antigens within the eye and that certain T-cell populations may be retained within the ocular space. This evidence of multiple antigen specificity and epitope spreading within the eye of a single patient will certainly pose difficulties when devising rational peptide therapy for uveitis.

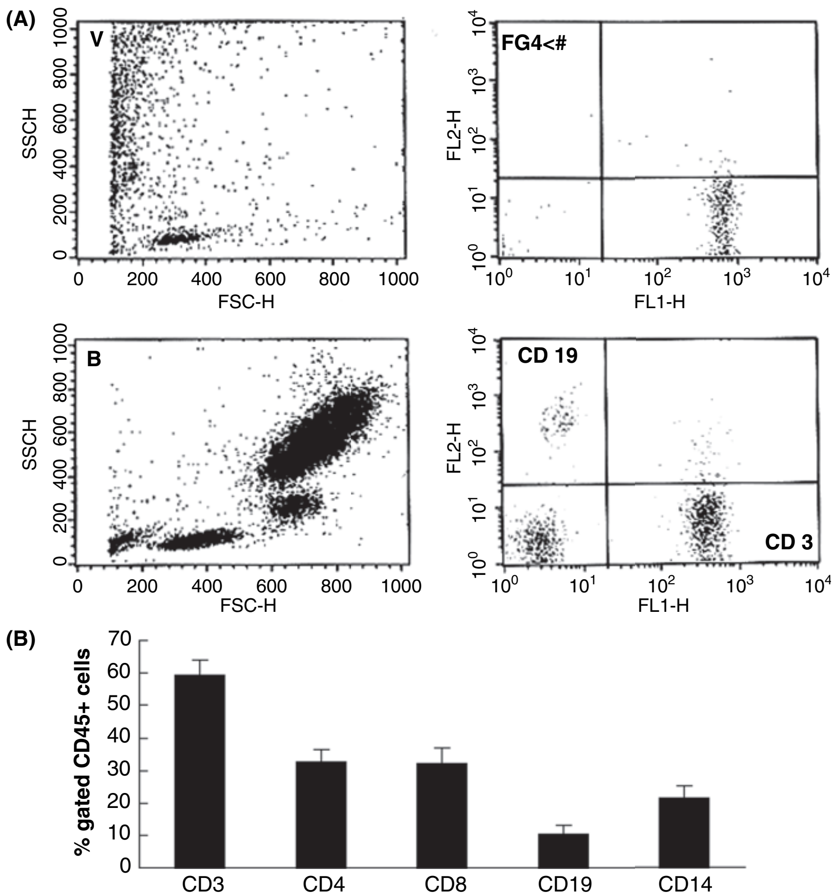

Figure 1 Phenotype of vitreous cells from patients with endogenous posterior uveitis. (**A**) Cells isolated from the vitreous (FACS dot plot V) are predominantly CD3+ T cells compared with blood (B). (**B**) Cumulative data from 7 patients. Data shown are means ± standard error. The majority of cells in the vitreous are CD3+ T cells with smaller numbers of B lymphocytes and monocytes also present.

EVIDENCE FOR REGULATION OF IMMUNE RESPONSES IN THE EYE DURING UVEITIS

To further study the function of vitreous T cells in uveitis we have attempted to clone these disease-associated T cells using IL-2 as a growth factor for activated cells in limiting dilution assay. However, clones could only be grown from 2 out of 11 vitreous samples. Clones generated were examined for cytokine secretion. Eight were found to secrete IFN-γ, and three secreted IL-4, showing a clear Th1 bias in responding cells. As discussed earlier, the normal eye has a number of mechanisms to maintain an immunosuppressive environment, restricting the

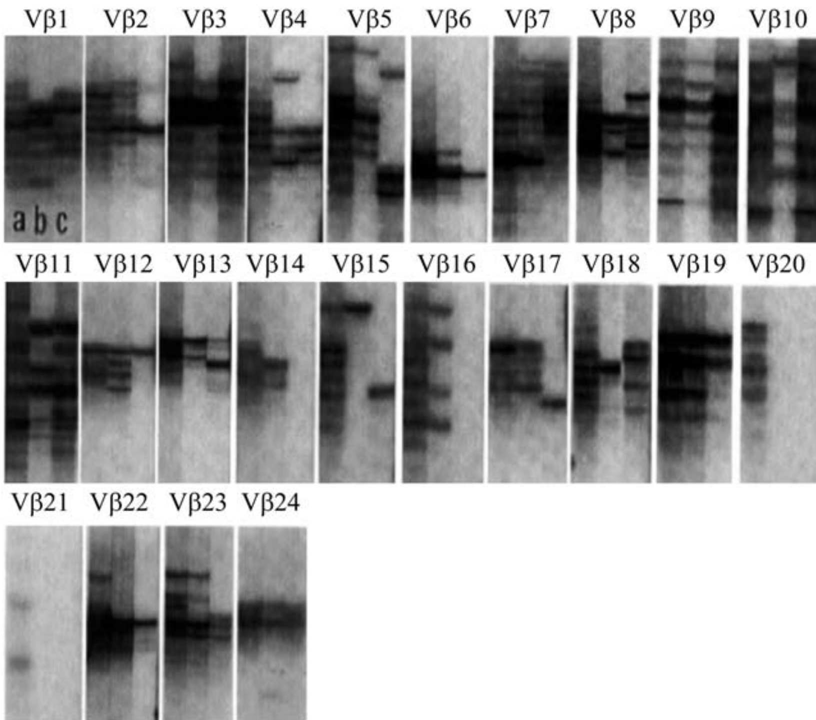

Figure 2 Evidence for multiple antigen specificity and epitope spreading in the response of a uveitis patient. CDR3 spectra-typing data of 22 TCR Vβ regions to show quantitative analysis of T-cell clones present within the peripheral blood (a), the vitreous (b), and the sub-retinal space (c). Monoclonality of response was evident in the sub-retina for Vβ6, Vβ12, Vβ15, Vβ17, and Vβ22, where only a single band was evident.

proliferation of activated T cells. These include Fas-Fas ligand induced apoptosis (36,37), and immunosuppressive cytokines such as TGFβ (1,2). Analysis of vitreous humor from uveitis patients and controls undergoing surgery (diabetes and cataract), showed that vitreous from uveitis patients contained high levels of sFas-ligand (150–1500 pg/ml), whereas control levels were undetectable (cataract) or <30pg/ml (diabetes). This finding, together with data from an anterior uveitis study that showed evidence for Fas-ligand–mediated apoptosis contributing to local immune regulation of ocular inflammation (38), provides a mechanism to explain the refractoriness of vitreous T cells to cloning and may account for the self-limiting clinical course of acute anterior uveitis. This hypothesis is also supported by our observation in EAU where FAS^{+} T cells in the vitreous were observed to undergo activation-induced cell death (39).

Apoptotic T cells also produce IL-10, and APCs taking up such apoptotic cells can induce tolerance rather than immunity (37). So why does chronic

inflammation persist in some uveitis patients? Genetically controlled immune dysfunction is thought to underlie persistent inflammation, and an imbalance in the production of other cytokines by ocular resident and infiltrating cells may continue to drive the immune response. Retinal pigment epithelial cells produce a wide range of both pro-and anti-inflammatory cytokines and other mediators in response to cytokine stimulation or contact with inflammatory cells (2), including TNF-α, IL-6, and IL-8 (40–42), as well as PGE_2, nitric oxide (NO), IL-15, and TGF-β (43,44). The outcome of any inflammatory response will therefore depend upon the balance of mediators present. Interleukin 15, IL-8, and PGE_2 all inhibit apoptosis by both stress-induced and Fas-ligand-induced pathways, potentially rescuing T cells from the effects of Fas ligation. Equally, uncontrolled production of excessive TNF-α and IL-1 will continue to drive damaging inflammation (45).

SUMMARY

This review highlighted data supporting the hypothesis that peripheral tolerance mechanisms for retinal S-antigen may be compromised. S-antigen can induce tissue DC maturation and migration, resulting in in vivo priming of immune responses in EAU. Although evidence for S-antigen peptide responsiveness in uveitis patients is accumulating, peptide therapy based on knowledge of pathogenic and regulatory epitopes on retinal antigens may not be practical, given the oligoclonal expansion and epitope spreading evident in patients. Therapeutic advances may lie in manipulation of APCs to induce tolerance. This could be directly through IL-10, or indirectly through administration of anti-pro-inflammatory cytokine reagents, such as TNF-α fusion proteins, allowing endogenous IL-10 or other suppressive factors to exert their effects, and allowing immunological homeostasis to be restored through generation of tolerance and regulatory cell populations (46–48).

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18

Role for Ocular Antigen-Presenting Cells in Pigmentary Forms of Glaucoma

Michael G. Anderson
Department of Molecular Physiology and Biology, University of Iowa, Iowa City, Iowa, U.S.A.

J. Wayne Streilein†
Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, U.S.A.

Simon W. M. John
The Jackson Laboratory, Howard Hughes Medical Institute, Bar Harbor, Maine, U.S.A.

INTRODUCTION

The glaucomas are a group of ocular diseases that affect approximately 70 million people worldwide (1). Hallmarks include the loss of retinal ganglion cells and their axons, morphological changes in the optic nerve head, and a characteristic pattern of visual loss (2–5). A key glaucoma risk factor involves intraocular pressure (IOP). IOP is elevated in most, but not all, forms of glaucoma (6,7). IOP is also the primary glaucoma-related factor that can currently be therapeutically manipulated (8,9). Unfortunately, the attempt to treat glaucoma by managing IOP is not always effective. We, and others, envision a future when improved patient outcomes might be achieved by using new therapeutic strategies that complement existing options. If this long-term goal is to be achieved, a fuller understanding of all forms of glaucoma pathogenesis will be necessary.

†Deceased.

HUMAN PIGMENT DISPERSION SYNDROME AND PIGMENTARY GLAUCOMA

Pigment Dispersion Syndrome (PDS) is an alarmingly common condition [2.45% of the general population (10)] characterized by an abnormal dispersal of iris pigment into the anterior chamber (AC) of the eye (11,12). A significant number of PDS patients (>10%) will develop elevated IOP and, ultimately, pigmentary glaucoma (PG) (13–17). The elevation of IOP in PG is sometimes explained as akin to a "clogging of the sink" effect whereby pigment accumulates in the trabecular meshwork and occludes aqueous humor (AqH) outflow. When AqH can't drain from the eye, IOP increases. However, experimental evidence has long suggested that the pathological response is actually more complex, likely including an ocular reaction to the pigment rather than a simple blockage due to the pigment itself (18–22).

THE DBA/2J MOUSE MODEL OF PG

DBA/2J mice develop a form of PG with similarities to human PDS/PG (23–26). By slit-lamp examination, indices of a pigment-dispersing iris disease in DBA/2J mice are first observable at 5–6 mo. The DBA/2J iris disease causes considerable pigment dispersion, which, like human PDS/PG, leads to pigment accumulations within the iridocorneal angle. Also similar to human PDS/PG, the DBA/2J iris disease is characterized by characteristic transillumination defects (Fig. 1A, B).

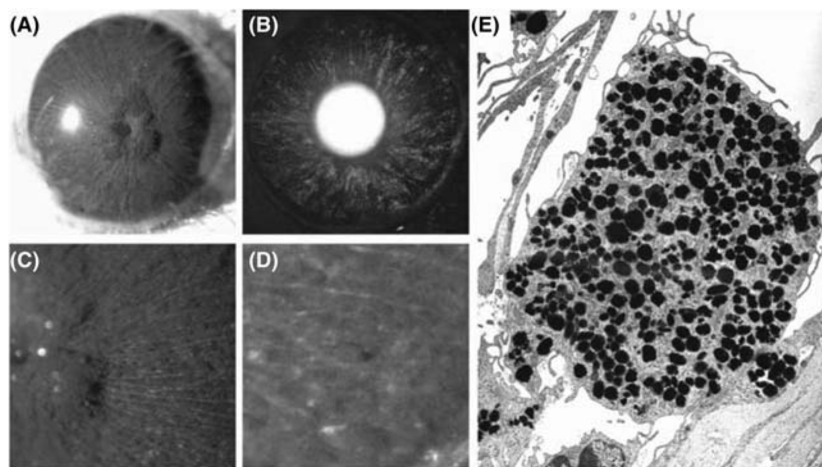

Figure 1 (*See color insert.*) Clinical presentation of DBA/2J iris disease. (**A**–**D**) Slit lamp images of the same 9-mo DBA/2J iris. (**A**) Pronounced peripupillary accumulations. (**B**) Transillumination defects. (**C**) Higher magnification highlights granular appearance of iris caused in part by the presence of antigen-presenting cells (APCs). (**D**) Electronically magnified image of a field from (**C**) showing a notably pigment-engorged cell in the center of the field. (**E**) Transmission electron micrograph of a pigment containing APCs within a DBA/2J anterior chamber.

By 8.5–10 mo, iris pigment dispersion is pronounced in DBA/2J mice and increased IOP is common. Thereafter, retinal ganglion cell and optic nerve degeneration ensue. Thus, DBA/2J mice can be utilized as a model of multiple glaucomatous phenotypes relevant for studying the pathogenic consequences associated with pigmentary forms of glaucoma (26–28).

Using functional genetic approaches, the iris disease-causing mutations have been identified for DBA/2J mice (23,24). Genetic experiments have clearly defined that pigment dispersal in DBA/2J mice results from the digenic interaction of mutations in two genes, tyrosinase-related protein 1 (Tyrp1) and glycoprotein (transmembrane)nmb (Gpnmb). The Tyrp 1 gene encodes a transmembrane melanosomal protein previously ascribed with both enzymatic and structural functions (29). Tyrp1 protein is believed to be present in all pigmented cells synthesizing melanin and would thus be expected to be present in both the iris stroma and the iris pigment epithelium. Less is known concerning Gpnmb. The Gpnmb gene is predicted to encode a heavily glycosylated transmembrane protein. Like Tyrp1, Gpnmb also encodes a protein known to be present in melanosomes (30,31). However, its role in melanosomes is relatively undefined. Interestingly, Gpnmb is also present in a small set of other cell types (32–34), notably including some types of antigen-presenting cells (APCs) (35,36).

Because Tyrp1 and Gpnmb are both present in melanosomes, a role for melanocytes seemed particularly likely. In order to test this hypothesis, we created hypopigmented strains of DBA/2J (23,24). Upon aging these mice, we found that reduced levels of pigmentation strikingly rescue the DBA/2J iris disease. Thus, at least one component of this iris disease involves potentially harmful molecules generated by melanocytes during the process of melanin synthesis. In this manuscript, we describe experiments addressing whether processes in addition to melanin synthesis also play a role in the DBA/2J iris disease, in particular, processes linked to APCs likely influenced by Gpnmb.

HINTS LINKING IMMUNE ABNORMALITIES TO PG

Could APCs also play a role in the DBA/2J iris disease? Gpnmb has previously been identified in some types of dendritic cells (DCs) (35,36) and the AC is known to harbor a robust population of these cells (37–39). Functionally, DCs are a sparsely distributed, migratory class of bone marrow-derived APCs that are specialized for the uptake, transport, processing, and presentation of antigen. DCs are essential for adaptive T-cell and B-cell mediated immunity (40), but also modulate innate responses via natural killer cells (41,42). Because DCs that present foreign antigen must also carry self-antigens, it is widely thought that DCs themselves are likely to also be responsible for tolerance, as well as immunity. Thus, if Gpnmb were influencing DCs, or other similar classes of ocular APCs, we hypothesized that the eyes of DBA/2J mice would likely exhibit immune abnormalities.

DBA/2J mice have been widely used in many different laboratories studying a variety of immune parameters. Although a few particularities of the DBA/2J immune system have been identified (43,44), the consensus has by large suggested

that DBA/2J are unnotably immunocompetent. However, by studying various DBA/2-related stocks housed and cryopreserved at The Jackson Laboratory, we have recently discovered that the Gpnmb^{R150X} mutation in DBA/2J mice was a spontaneous mutation first known to have become fixed in the stock in 1995 (at least fixation occurred between late 1980s and 1995) (23,26). Previous to this time, DBA/2J mice carried either wild-type Gpnmb alleles, or were segregating for the mutation. Thus, any characterization of immune phenotypes conducted with DBA/2J mice prior to 1995 might well have not been able to detect abnormalities associated with Gpnmb. Therefore, we decided to perform a characterization of the DBA/2J ocular immune microenvironment and to specifically look for any indices of potential immune involvement in DBA/2J glaucoma.

IMMUNE STATUS OF EYES FROM DBA/2J MICE

The AC is an immune privileged site (45). Conceptually, it is useful to consider immune reactions of the eye as a delicate balance between two potentially conflicting needs; the need to provide immune protection against pathogens while simultaneously also maintaining a clear ocular media needed to allow light images to fall accurately on the retina. Thus, immune protection of the eye occurs in a manner that is largely devoid of immunogenic inflammation. Many factors undoubtedly contribute to the immune privileged status of the AC. Experimentally, the manifestation of ocular immune privilege involves several features that can be measured, including: (*i*) the existence of an immunosuppressive microenvironment, (*ii*) the presence of an intact blood–ocular barrier, and (*iii*) the induction of tolerance to eye-derived antigens. Therefore, characterization of these three parameters is a meaningful manner for surveying whether eyes of DBA/2J mice harbor any ocular immune abnormalities.

Immunosuppressive Microenvironment

Several factors render the intraocular microenvironment particularly immunosuppressive, including AqH. Normal AqH strongly suppresses activation of a wide variety of cells in vitro, and profoundly inhibits T-cell activation. In contrast, DBA/2J AqH from mice of all ages tested (2–10 mo) consistently failed to inhibit T-cell proliferation, and actually exhibited mitogenic activity (46). Interestingly, even AqH from 2-mo DBA/2J lacked immunosuppressive properties. This age precedes clinical indices of pigment dispersion, perhaps suggesting that immune abnormalities may indeed play a very early role in aspects of the DBA/2J glaucoma.

Blood: Ocular Barrier

Another experimentally measurable factor contributing to ocular immune privilege is the presence of anatomically derived immune barriers. As a result of an intact blood–ocular barrier, normal AqH contains extremely low levels of protein and no leukocytes. AqH from 2-mo DBA/2J eyes contained barely detectable

amounts of protein (46). However, AqH from 4-mo DBA/2J mice contained slightly elevated levels of protein and these levels rose progressively through all ages tested. Leukocytes were first detectable in DBA/2J AqH at 6 mo (46). At 6 mo, the predominant leukocytes present were neutrophils, and by 7 mo, mononuclear cells predominated. These results were somewhat surprising given that by slit-lamp examination, DBA/2J eyes lack notable indices of redness or severe inflammation. However, these data clearly demonstrate that leukocyte infiltration is indeed an early feature of the iris disease that gives rise to DBA/2J glaucoma.

Interestingly, the most abundant infiltrating leukocytes at 7 mo were macrophage-like cells containing melanin (Fig. 1C–E). Histologically, these pigment-engorged cells were present in the AC and were particularly notable in the iris stroma near the pupillary border. FACS® (BD FACSCalibur™, BD Biosciences, San Jose, California, U.S.A.) analysis of AqH revealed that these cells stained positive for CD11b and MHC class II (some were additionally positive for CD11c), consistent with the phenotype of macrophage and DC type cells. For clarity, we will refer to these cells here simply as APCs.

ACAID

Ocular immune privilege expresses itself in part via a form of tolerance to eye-derived antigens termed ACAID (anterior chamber-associated immune deviation). Antigenic material encountered in the AC elicits a deviant form of systemic immunity that is devoid of effectors causing immunogenic inflammation (T cells that mediate delayed hypersensitivity and B cells that secrete complement-fixing antibodies). Thus, if an antigen such as ovalbumin is injected into the AC, the recipient mice fail to acquire OVA-specific delayed hypersensitivity upon subsequent challenge. OVA injections into the AC of 2-mo DBA/2J mice moderately impaired the development of OVA-specific delayed hypersensitivity, whereas injections into older 4- and 6-mo DBA/2J mice failed completely to inhibit OVA-specific delayed hypersensitivity (46). The lack of functional ACAID in DBA/2J mice represents a striking deficit in ocular immune function. Furthermore, the time frame is again consistent with the suggestion that immune abnormalities precede the onset of clinically observable disease.

CAUSATION VERSUS COINCIDENCE

The above experiments indicate that DBA/2J eyes exhibit several previously unrecognized phenotypes suggestive of ocular immune abnormalities. They support an effect of the Gpnmb mutation on immune phenotypes. We next wished to test whether these abnormalities could be additionally linked to the DBA/2J glaucoma and the genotype of bone marrow-derived cells by analyzing bone marrow chimeras. Bone marrow contains progenitors for hematopoietic lineages, causing reconstituted recipient mice to develop leukocyte lineages with cells of the donor genotype. Therefore, if bone marrow-derived lineages, such as DCs, play a role in DBA/2J

ocular phenotypes, it should be possible to modify those phenotypes via generating radiation-induced bone marrow chimeras. We tested this hypothesis by using bone marrow from B6D2F1 mice (which have normal irides and do not develop pigment dispersion or glaucoma) as donor material for irradiated DBA/2J recipients (46). Cohorts of these mice were subsequently aged and characterized to address whether the eyes of these chimeric mice displayed immune abnormalities similar to those in unmodified DBA/2J mice, and whether the B6D2F1 bone marrow cells could ultimately influence the manifestation of pigment dispersion.

Cohorts of D2 mice were reconstituted with either B6D2F1 (hereafter referred to as chimeras) or DBA/2J (control) bone marrow and characterized (46,47). As expected, the control mice that were reconstituted with DBA/2J bone marrow developed a pigment-dispersing iris disease with all of the same features as unmanipulated DBA/2J mice. In contrast, the chimeras reconstituted with B6D2F1 bone marrow exhibited a striking rescue of AC phenotypes, including marked reduction of transillumination defects, a lack of iris pigment dispersion, and an absence of AC enlargement. These findings indicate that not only has the iris disease been suppressed, but that the progression toward glaucoma has been stopped as well.

To further characterize the rescuing effects of B6D2F1 marrow on DBA/2J recipients, we also characterized the immune status of eyes from these mice (46). At six months, the AqH from chimeric mice contained no leukocytes and barely detectable levels of protein. Eyes of these mice also robustly supported ACAID induction. Thus, the form of anterior uveitis typical of unmanipulated DBA/2J mice did not occur in these chimeras. In sum, these experiments with DBA/2J bone marrow chimeras indicate that bone marrow-derived lineages are required for the full pathogenesis of the DBA/2J form of PG. The restoration of ACAID-induction capacity via wild-type B6D2F1-derived APCs supports the notion that abnormal APC function contributes to the pathogenesis of PDS/PG.

These experiments with DBA/2J chimeras also generated a few additional findings that impact our understanding of this disease. First, not all of the ocular immune abnormalities typically observed in DBA/2J mice were corrected by bone-marrow transfers; the AqH of chimeric mice still failed to suppress T-cell activation in vitro (46). This result indicates that DBA/2J mice evidently harbor multiple ocular immune abnormalities. Some of these appear closely related to the pigment-dispersing iris disease (loss of ACAID, breakdown of the blood–ocular barrier) and others appear to be completely independent (ability of the AqH to suppress T-cell activation). The factors causing this abnormality of DBA/2J AqH remain unknown. Second, reciprocal bone marrow transfers were simultaneously conducted (DBA/2J bone marrow was transferred into lethally irradiated B6D2F1 recipients) but failed to transfer iris disease (46). A simple interpretation of this experiment is that a melanosomal defect must first be present to initiate an iris disease that is subsequently propagated by the immune reactions. Because the B6D2F1 mice do not exhibit the melanosomal defects of DBA/2J mice, the ability to observe subsequent immune reactions is curtailed.

One of the striking themes arising from these experiments is that ocular immune abnormalities and APCs are intimately related to the form of PG occurring in DBA/2J mice. The form of inflammation occurring in DBA/2J mice involves a breakdown of the blood–ocular barrier, an infiltration of leukocytes including mononuclear macrophage-like cells engulfing pigment, and a loss of the ability to support ACAID (46). The finding that bone marrow-derived lineages can rescue both these ocular immune abnormalities and the clinical indices of the glaucomatous iris disease itself indicates that these events are a primary disease characteristic and not merely a secondary consequence of a diseased iris.

A variety of groups have previously demonstrated that immunity and pigmentation can exhibit interesting interactions. For example, melanin exhibits potent adjuvant-like properties and enhances inflammation in experimental autoimmune uveitis (48,49). Given the significant amount of melanin that is ingested via ocular APCs during the pigment-dispersing iris disease of DBA/2J mice, it would be interesting to determine whether this ingested pigment degrades the ACAID-inducing capacity of ocular APCs. If true, perhaps this loss of ACAID potential promotes inflammatory disease-causing conditions by making it possible for a form of autoimmunity against melanin-related molecules to arise. Possibly arguing against this, ACAID was defective in DBA/2J eyes at ages before there was prominent pigment dispersion.

CONCLUSION

Much remains to be learned regarding the mechanisms linking these ocular immune abnormalities, APCs, and pigment dispersion. In particular, it will be important to continue to better characterize the cellular expression of Gpnmb in the eye and bone marrow-derived lineages. It is tempting to speculate that Gpnmb may well be expressed in ocular DCs and influence the ability to support ACAID. Experiments testing this hypothesis in mice are currently underway. Whether regulated by Gpnmb, or by other genetic factors yet to be identified, the realization that APCs may influence the progression of pigmentary forms of glaucoma is an important finding. Perhaps most significantly, these experiments in mice have suggested a new hypothesis that can now be examined in human PDS/PG patients. In the long term, these findings may contribute to new therapeutic interventions capitalizing on immune-based interventions in PDS/PG management.

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19

Association of Major Histocompatibility Class II Antigens with Core Subdomains Present Within Human Ocular Drusen

Gregory S. Hageman and Robert F. Mullins
Department of Ophthalmology and Visual Science,
University of Iowa, Iowa City, Iowa, U.S.A.

INTRODUCTION

Age-related macular degeneration (AMD) is an ocular disease that affects millions worldwide. Approximately 15% of individuals affected with AMD develop severe forms of the disease—referred to as choroidal neovascularization and geographic atrophy—that typically lead to functional blindness. One of the earliest detectable risk factors for AMD is the presence of drusen, extracellular deposits that form between the retinal pigment epithelium (RPE) and Bruch's membrane. The density, number, and size of drusen are significant risk factors for both geographic atrophy and choroidal neovascularization (1). Thus, there has been a strong recent interest in the characterization of drusen composition with the hope that a better understanding of drusen development might provide new insights into specific biological pathways involved in the development of AMD.

Over the last several years there has been emerging evidence that inflammation plays a crucial role in drusen biogenesis and the development of AMD (2–4). A number of studies have shown that a host of molecules involved in immune-mediated processes, including complement components, terminal complement complexes, clusterin, and vitronectin (5–9) are present in drusen. Interestingly,

a number of drusen-associated molecules are synthesized locally (5,8,10), although the relative contributions of locally- and vascular-derived sources for drusen components is not clear. Some molecules, such as amyloid P component, for example, are abundant in drusen but do not appear to be synthesized locally (8), suggesting that they likely accumulate within drusen following their extravasation from the choriocapillaris. A role for immune-mediated processes in drusen formation is further supported by the observation that ocular drusen develop at a relatively young age in individuals afflicted with inflammatory renal disease (11–13).

Drusen also possess core-like domains that are labeled by peanut agglutinin (PNA) following neuraminidase digestion. Thus, these domains appear to be comprised of glycoproteins possessing O-linked carbohydrates (14). We also noted recently that human leukocyte antigen (HLA)-DR antibodies react with many drusen, often restricted to central core domains (8), similar in size and location to those labeled with PNA. This finding was of interest, as it suggested that cells or cell-derived membranes might be components of drusen. Although increased levels in HLA-DR immunoreactivity of retinal microglia have been documented in AMD donor eyes (15), and stellate HLA-DR-expressing cells in the rat and human choroid have been described (16), little is known of the choroidal distribution of HLA-DR in normal or aging eyes—particularly at the RPE-choroid interface—or the cells that express them in the choroid. We describe herein the patterns of HLA-DR in drusen and Bruch's membrane, with an emphasis on the association of HLA-DR with PNA-binding drusen core domains in aging human eyes.

DISTRIBUTION OF HLA-DR IN DRUSEN

In order to assess the potential relationship between MHC class II antigens and PNA-bound core domains, we performed dual labeling experiments using PNA (following neuraminidase digestion) and two monoclonal antibodies directed against HLA-DR (Dako clones CR3/43 and TAL.1B5). The posterior eyecups of human donor eyes (collected within 5 hours of death), comprised of retina, RPE, choroid, and sclera, were either preserved in paraformaldehyde-containing fixative (5) or embedded directly without fixation. Processing, preparation, and sectioning of human eyes, including enzymatic digestion and immunohistochemical staining, were performed as described previously (5,14). HLA-DR distribution was detected using either biotinylated secondary antibodies followed by avidin-conjugated peroxidase (Vector Universal ABC kit, Burlingame, CA) and Vector VIP substrate, or Alexa-488-conjugated (Molecular Probes, Eugene, OR) or Cy3-conjugated (Chemicon, Temecula, CA) secondary antibodies.

Labeling of drusen with PNA is largely confined to central regions within drusen following removal of terminal sialic acid residues, as described previously (14). HLA-DR immunoreactivity is frequently localized to the same core domains as those bound by PNA (Fig. 1). The diameters of HLA-DR domains are typically larger than those bound by PNA, however, suggesting that there may be partitioning within these unique drusen subdomains (Fig. 1). In some cases, a central,

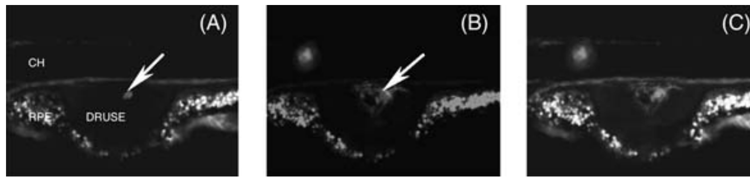

Figure 1 (*See color insert.*) Light micrographs depicting the spatial relationship between drusen core domains labeled by both peanut agglutinin (PNA) and human leukocyte antigen (HLA)-DR antibody. (**A**) Image of PNA labeling following prior treatment of the section with neuraminidase. Note the labeling of a drusen-associate core domain (*arrow*) and the inter-photoreceptor matrix and the endogenous autofluorescence of Bruch's membrane. (**B**) Same field as that depicted in panel (**A**), showing HLA-DR (CR3/43 clone) labeling. Note the voluminous drusen core domain and leukocyte. (**C**) Merged image of panels (**A**) and (**B**). Note that RPE lipofuscin is highly autofluorescent in both the fluorescein and rhodamine channels. The HLA-DR reactive core domain overlaps, and extends beyond, the boundary of the PNA-bound drusen core domain.

intensely stained core of HLA-DR immunoreactivity, surrounded by a diffuse, less intense halo of HLA-DR reactive material is observed (Fig. 2A). HLA-DR-reactive core domains are often contiguous—via a narrow bridge that spans Bruch's membrane—with distinct HLA-DR positive choroidal cells (Fig. 2B).

Multiple patterns of drusen-associated HLA-DR immunoreactivity are observed in different donor eyes. Some drusen, particularly those of the "hyaline" phenotype, which appear homogeneous by light microscopy, generally do not possess obvious HLA-DR immunoreactivity (not shown). Non-hyaline drusen possess HLA-DR reactive cores, as described above (Fig. 3A, B); this pattern is noted in

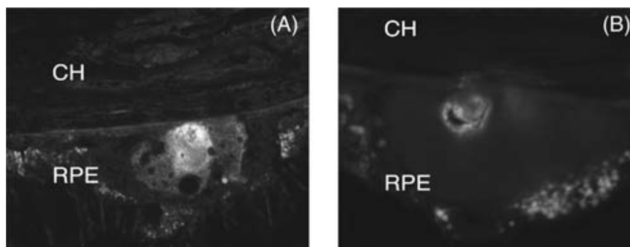

Figure 2 (*See color insert.*) Light micrographs showing drusen-associated patterns of human leukocyte antigen (HLA-DR) immunoreactivity. (**A**) In many cases, intense HLA-DR (green) immunoreactivity is associated with a central, core-like domain that is surrounded by a more diffuse circumferential reaction product. (**B**) HLA-DR antibody (red) also reacts with choroidal cells that are connected to HLA-DR reactive drusen core domains via distinct bridges that span Bruch's membrane. *Abbreviations*: CH, choroid; REP, retinal pigmented epithelium.

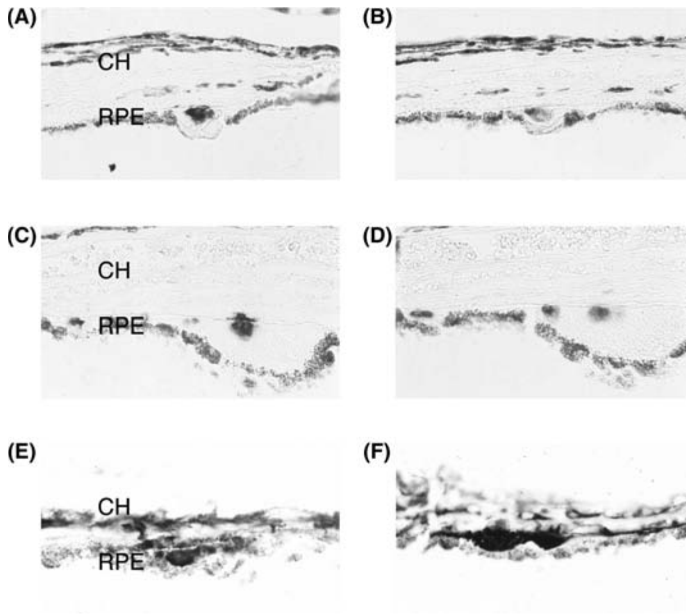

Figure 3 Light micrographs depicting various patterns of drusen immunoreactivity with human leukocyte antigen (HLA)-DR antibodies [monoclonal antibodies TAL.1B5 (**A, C, E**) or CR3/43 (**B, D, F**)]. The same regions are generally detected with different monoclonal antibodies. In some drusen, typically those of the "hyaline" phenotype, no immunolabeling with HLA-DR is observed (not shown). Labeling to core domains in both small (**A, B**) and large (**C, D**) drusen is frequently observed. Immunoreactive patches are sometimes in the choroid and/or in Bruch's membrane detected adjacent to drusen, as shown in (**C**). These patches frequently correspond to choroidal cell bodies, based on colocalization with nucleus specific probes (data not shown). In some cases, drusen are completely HLA-DR immunoreactive (**E, F**). *Abbreviations*: CH, choroid; REP, retinal pigmented epithelium.

both small and large drusen (Fig. 3C, D). Entire drusen are HLA-DR positive in some cases (Fig. 3E, F); these are typically very small, subclinical drusen.

DISTRIBUTION OF HLA-DR IN THE CHOROID AND SCLERA

Numerous HLA-DR positive cells are also observed within the sclera and throughout the choroidal stroma. These cells are typically attenuated on cross section (Fig. 4) and generally oriented parallel to the scleral and choroidal lamellae. Some dendritic processes arising from these cells are apparent. Labeling of the choriocapillaris with HLA-DR antibodies is also noted in some cases (Fig. 4), although there is considerable donor-to-donor variation. In an examination of over 60 fixed eyes, the choriocapillaris was labeled with HLA-DR in only two donors. Interestingly, both of these donors had a clinical history of age-related macular degeneration (data not shown).

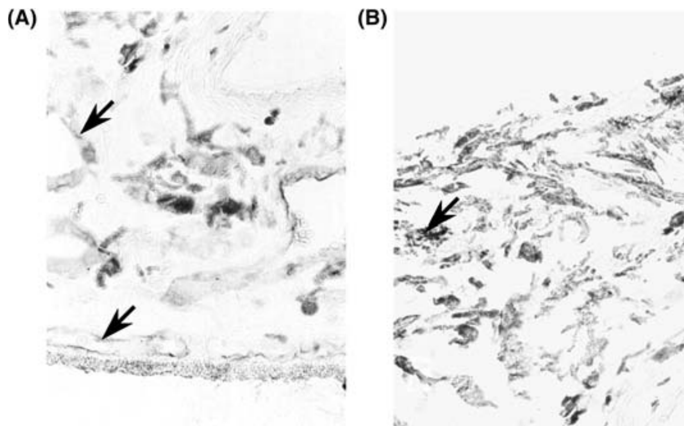

Figure 4 Light micrographs showing the distribution of human leukocyte antigen (HLA)-DR-positive (CR3/43 antibody) cells in the choroid of an eye derived from a 49-year-old female. In some cases, the choriocapillaris and some large vessels are immunoreactive (**A**, *arrows*). In the same eye (**B**), nonpigmented, choroidal cells are labeled with HLA-DR antibody (*arrow*).

DISCUSSION

We have shown previously that PNA labels core-like domains within drusen. We postulated that these domains may indicate an early stage in the biogenesis of some drusen (14). In the current study we show that both PNA and HLA-DR colocalize to the same drusen core domains. One explanation of this colocalization is that a portion of PNA labeling may be associated with O-linked carbohydrate residues on HLA-DR molecules. It is also notable, in this context, that HLA-DR associated with lymphoblastoid cells possesses penultimate PNA-binding sites that are masked by sialic acid (17). Alternatively, PNA and HLA-DR antibodies may not react with the same core domain-associated molecules, since the patterns of PNA binding and HLA-DR immunoreactivity only overlap partially. It appears that core domains within drusen possess a partitioning of molecules that has not been appreciated previously.

The observation of HLA-DR residues within drusen suggests a potentially important role of immune-associated events in the biogenesis and/or biology of drusen. Thus, it will be important to identify the source(s) of HLA-DR molecules in drusen in order to understand more clearly the pathways involved in drusen biogenesis and the etiology of drusen-associated diseases such as age-related macular degeneration. It has been documented that the RPE expresses HLA-DR in vitro following administration of interferon gamma (18) and in vivo in diseases such as retinitis pigmentosa (19). Thus, it is feasible that the RPE, which synthesizes a number of drusen-associated molecules, also synthesizes HLA-DR and exports membrane-bound HLA-DR into developing drusen. Along these lines, we

have detected HLA-DR transcripts in RPE from human donor eyes using RT-PCR (8), although we have not typically observed HLA-DR immunoreactivity of RPE cells in situ. It is difficult in this scenario, however, to explain the restricted distribution of HLA-DR to core-like domains within the majority of drusen. It might explain the cases in which HLA-DR immunoreactivity is observed throughout smaller subclinical drusen, suggesting that RPE-exported HLA-DR might serve as an early nidus for drusen formation.

The localization of HLA-DR in drusen cores raises the more likely possibility that it is derived from, or associated with, choroidal cells (9). This is consistent with images showing continuity between HLA-DR immunoreactive drusen core domains and distinct HLA-DR reactive via a connecting “bridge” that breaches Bruch’s membrane. Recent analyses indeed suggest that many of these HLA-DR reactive cells are indeed choroidal dendritic cells that possess cellular processes that extend through Bruch’s membrane and terminate as spherical “core” domains within drusen. If future quantitative studies document an association of dendritic cells with drusen biogenesis, it will be important to determine the role of these cells in the etiology of drusen-associated diseases such as age-related macular degeneration, the leading cause of irreversible blindness in the world.

Along these same lines, this study documents a variable distribution of HLA-DR reactive cells in the sclera, choroid, and choriocapillaris amongst donors, suggesting that their presence may be associated with an as-yet unestablished phenomenon. Thus, it will be important in future studies to determine the distribution and role of choroidal dendritic cells in ocular homeostasis and disease. Since this study shows that HLA-DR labeling is sensitive to fixation, this issue will have to be considered in the design of future studies directed toward the distribution and quantitative assessment of HLA-DR immunoreactive cells in the choroid and sclera.

In summary, we present findings related to the distribution of antigen-presenting cells in the human sclera and choroid, with emphasis on the multiple patterns of HLA-DR immunoreactivity in drusen. The partial overlap of PNA-binding saccharides in drusen and of HLA-DR suggests an association between immune cells and drusen cores, and, by extension, a possible role for these cells in drusen biogenesis. We suggest that a more thorough understanding of HLA-DR in age-related macular degeneration, including genotypes that may dispose individuals to more severe or earlier onset forms of the disease, will provide a more complete understanding of the role of immune-mediated processes in this blinding disease. MHC genotypes have been found to affect the risk of a number of several age-related diseases (20), including some retinopathies (21,22). A recent study suggests that these alleles are associated with age-related macular degeneration (23). Based on the association of HLA-DR molecules with drusen, it is plausible that specific HLA genotypes have a similar effect on the risk of developing AMD. This issue is being addressed in ongoing investigations.

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20

Role of Macrophages in Uveal Melanoma

Martine J. Jager
Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands

Teemu Mäkitie, Päivi Toivonen, and Tero Kivelä
Department of Ophthalmology, Helsinki University Central Hospital, Helsinki, Finland

INTRODUCTION

Uveal melanoma is the most common primary intraocular malignancy in adults, with an estimated annual incidence of 4 to 11 cases per million subjects in Caucasians (1–3). In contrast to cutaneous and conjunctival melanoma (4), the incidence has been stable or may even be decreasing in some populations (2,3).

Uveal melanomas that arise from melanocytes of the choroid and, especially, the ciliary body, are relatively slow-growing tumors but metastasize often. In a very long-term follow-up study of 289 patients treated during the era in which all patients with uveal melanoma underwent enucleation, melanoma-related mortality was 31% at 5 years, 45% at 15 years, and 49% at 25 years, estimated with cumulative incidence analysis, which appropriately takes into account competing causes of death (5). Between 15 and 35 years after enucleation, 20% to 33% of all deaths were still due to metastatic uveal melanoma (5).

These data indicate that metastases may remain in the body for a very long period prior to growth, which is known as dormancy. At least two factors likely play a role. First, the tumor cells themselves may have specific characteristics which allow them to remain “dormant,” but it is unknown what determines when the cells “wake up.” Secondly, the immune system may inhibit tumor cell growth

for a prolonged period of time. We do not know which of the two is the most relevant, or whether both of them are equally important.

Whereas further research is necessary to solve the problem of dormancy, it is clear that during the phase of hematogeneous dissemination, immunological factors play a role. Immune responses that evolve at this stage may still influence tumor behavior years later. Similar to immunity after viral disease, tumor exposure may lead to life-long immunity. We describe parameters that are related to the development of metastases and then focus on one of the factors, macrophages, which are known to infiltrate the tumor.

PROGNOSTIC FACTORS IN UVEAL MELANOMA

Many factors related to the patient and the tumor have an influence on survival (6,7). In the very long-term study mentioned, clinical variables associated with a significantly higher incidence of melanoma-related death were involvement of the ciliary body [hazard ratio (HR) 1.9], extraocular extension (HR 2.3), and large basal diameter of the tumor (HR 1.1 for each mm increase) (5). When competing causes of death were taken into account, the effect of age was smaller but the effect of gender was higher than traditionally estimated: females were 1.4 times more likely to die of uveal melanoma than men.

A number of histopathological characteristics are associated with death from uveal melanoma. These include, but are not limited to, presence of microvascular loops and networks [HR 1.4 for each category change in one population-based data set (8)], presence of epithelioid cells (HR 2.9), microvascular density (HR 1.3 for each unit change), and mean diameter of the ten largest nucleoli (HR 1.4 for each μm increase), which are independently associated with prognosis (9–15). A particularly strong prognostic factor is presence of a characteristic chromosome abnormality, monosomy 3, in tumor tissue (16,17).

Two different biological principles may be influenced by these parameters: one is easier dissemination of tumor cells from the eye, which is associated with the proportion of patients who develop metastasis, and the other is faster growth of the tumor cells, which is associated with a shorter time to death (5,18).

If the immune system is involved, expression of immunologically relevant molecules should be associated with prognosis. This is indeed the case: Blom et al. observed that high expression of HLA-A and HLA-B was related to shorter survival (19). This suggested that shedding of uveal melanoma micrometastases with a low expression of HLA class I into the systemic circulation may facilitate their removal and prevent the development of metastases. Later studies have supported these findings. The association between a high HLA class I antigen expression and short survival was confirmed in a series of 46 primary uveal melanomas (20): tumors that had metastasized or developed extraocular extension were more often immunopositive for HLA class I and class II antigens (21), and only 18% of spindle cell melanomas but 82% of epithelioid cell melanomas, which are associated with better and worse prognosis, respectively, reacted with an antibody to HLA-A (22).

The association with high HLA class I expression with melanoma-related death can be explained by removal of low-HLA class I antigen-expressing tumor cells from the bloodstream by natural killer (NK) cells. It is likely that NK cells attack tumor cells when in transit from the eye to the extraocular sites, such as the liver. This hypothesis is supported by experimental studies from the group of Niederkorn: in mice, melanoma cells with a low HLA class I expression did not induce metastases when injected into the bloodstream, while melanoma cells with a high expression gave rise to a high number of liver metastases (23).

PRESENCE OF LYMPHOCYTES AND MACROPHAGES IN PRIMARY UVEAL MELANOMA

Tumor-infiltrating lymphocytes and macrophages have long been known to reside in uveal melanoma (24–28). In 1977, Davidorf and Lang evaluated lymphoplasmocytic infiltration, which was graded intense in 5% and moderate in 12% of 326 primary uveal melanomas, and they found it to be unrelated to survival (24). By light microscopy, the number of macrophages in primary uveal melanoma was graded low in 89% of 1526 large choroidal melanomas (28).

Tobal and coworkers used immunohistochemistry to characterize infiltrating cells of 16 uveal melanomas in 1993, and found high numbers of T cells and macrophages in 7 (26). Most T cells were activated $CD8^+$ cells. In a concurrent study, Whelchel et al. reported infiltration of primary uveal melanomas by T cells to be associated with higher mortality (29). In 1996, De Waard-Siebinga et al. (30) found small numbers of infiltrating lymphocytes in each of 24 primary uveal melanomas, with a predominance of T cells, including $CD3^+$ and $CD4^+$ cells. Furthermore, CD11b immunoreactivity characteristic of monocytes, macrophages, and granulocytes was seen in 20 melanomas. The same pattern was seen with mAb Leu-M3, directed against the CD14 epitope, except for one case (30). One tumor showed necrosis following irradiation prior to enucleation, and the infiltrating cells in this tumor were CD11b negative/CD14 positive. A significant correlation was observed between the presence of CD11b positive cells and expression of HLA Class I (30).

Mäkitie et al. (31) studied the presence of tumor-infiltrating macrophages in choroidal and ciliary body melanoma in relation to survival. They used the CD68 epitope as a marker for macrophages, and found high numbers of immunopositive cells to be present in 32% and moderate numbers in 51% of 139 primary tumors. They evaluated three monoclonal antibodies to the CD68 epitope and found that all but one, mAb PG-M1, cross-reacted with uveal melanoma cells (31). Photographs were used to standardize evaluation of the number and type of macrophages; their morphology varied from round through intermediate to dendritic.

The presence of moderate to high numbers of macrophages was related to large basal diameter of the tumor, the presence of epithelioid cells, heavy pigmentation, and a high microvascular density (31). The association between

increased numbers of macrophages and the presence of epithelioid cells and large basal diameter was also seen in the Collaborative Ocular Melanoma Study (28). Mäkitie did not find a statistical association between macrophages and microvascular loops and networks in his series, but observed that macrophages were often arranged along these and other microvascular patterns (31). Clarijs et al. subsequently confirmed the presence of macrophages in and around the matrix sheets that constitute microvascular loops and networks (32).

Mäkitie observed that the number of macrophages was related to survival (31). The ten-year cumulative probability of survival was 0.90 for patients with few, 0.58 with moderate numbers and 0.43 with many CD68$^+$ cells: tumors with few immunopositive cells gave a better prognosis than those with a moderate and high number of immunopositive cells (31). The different morphological types were not related to survival.

POTENTIAL ROLE OF MACROPHAGES IN TUMOR IMMUNOLOGY OF PRIMARY UVEAL MELANOMA

One way of inducing systemic immune responses is intradermal or intraperitoneal injection of antigen-presenting cells (APCs), which carry the appropriate antigen. It is imaginable, that a similar mechanism might occur naturally. If APCs, such as macrophages and dendritic cells, are present inside a tumor, it might be that they are carried along when tumor cells are released into the bloodstream. Should this occur, it is possible that the mixture could induce a systemic immune response.

Another way of immunization could be caused by extraocular extension of the tumor. Injection of an antigen under the conjunctiva induces a strong systemic immune response against that specific antigen. Furthermore, in case of extraocular extension, tumor cells may be transported by dendritic cells to regional lymph nodes and, even when the material is insufficient to cause a local metastasis, the presence of tumor antigens might induce systemic T- or B-cell responses.

ANIMAL MODELS FOR SYSTEMIC IMMUNIZATION FROM A PRIMARY INTRAOCULAR TUMOR

Ocular immune privilege permits the outgrowth of tumors that would be rejected when placed elsewhere in the body. Schurmans et al. (33,34) developed a model in which melanoma cells transformed by the early region 1 of human adenovirus type 5 (Ad5E1A) are implanted in the anterior chamber of an immunocompetent C57Bl/6 mouse. The tumor first grows to fill the anterior chamber, but is subsequently rejected and disappears. In immunodeficient nude mice, however, the tumor is lethal (33,34). Since the tumor is removed from the eye in normal mice, a T-cell dependent rejection mechanism is involved.

Analysis of the molecular and cellular mechanisms involved in tumor rejection showed that perforin, TNF-α, Fas ligand, MHC class I, and CD8$^+$ cells

did not play a role in this model. Tumor rejection was, however, dependent on $CD4^+$ cells, in relation with MHC class II (34). The $CD4^+$ cells were able to remove the tumor without causing phthisis. We hypothesized that, if there is an immune response, sensitization must take place. Intraocular macrophages, dendritic cells, or both may carry tumor-associated antigens from the intraocular tumor to the regional lymph nodes and thus stimulate a systemic T-cell response (34).

Boonman et al. (35) showed by adoptive transfer of CFSE-labeled, E1A-specific TCR-transgenic T cells that tumor-related antigens in another intraocular Ad5E1A-induced tumor model indeed do drain into the ipsilateral submandibular lymph nodes. If these transgenic T cells encounter E1A antigen, they start to proliferate. After each cell division, the amount of CFSE in the T cell is halved, and the decrease in CFSE expression can be determined by FACS analysis. No dividing cells were detected in the spleen, indicating that not enough antigen was carried there to induce an immune response (35). The presence of a tumor-specific endogenous response was shown by applying E1A/Db tetramers. Again, $CD8^+$ cells could be found in the submandibular lymph nodes, but not elsewhere in the body.

These data indicate that, in the mouse, the presence of an intraocular tumor induces a local immune response, although, in the latter model, the intraocular tumor did not regress. In this regard, the latter model appears to show much similarity to uveal melanoma in humans. Interestingly, complete depletion of macrophages led to progressive tumor growth in this model: it is clear that in the animal model, macrophages are vital for intraocular tumor eradication (36).

WHAT DETERMINES THE PRESENCE OF MACROPHAGES IN PRIMARY UVEAL MELANOMAS?

One can wonder whether some specific mediators produced by uveal melanoma cells attract macrophages or prevent them from leaving the tumor once they have been recruited.

Clarijs et al. (37) examined the role of endothelial monocyte-activating polypeptide II (EMAP-II) in the recruitment of macrophages to uveal melanoma. EMAP-II is a cytokine present both in normal and malignant tissues, which has the ability to attract monocytes and granulocytes. Hypoxic and apoptotic cells release EMAP-II, thereby causing macrophage influx, which may lead to macrophage-induced angiogenesis in hypoxic areas (38,39).

EMAP-II expression was detected in 23 of 25 primary uveal melanomas; in eight tumors, all melanoma cells expressed EMAP-II (37). Macrophages were more abundant in areas with EMAP-II. As mentioned, $CD68^+$ macrophages often are found closely associated with periodic acid-Schiff (PAS) positive microvascular loops and networks, extravascular structures that incorporate microvessels and, possibly, conduct fluid themselves (37,40). In the 15 tumors with local differences in EMAP-II expression, EMAP-II was especially strong

in areas of loops and networks, and in areas with necrosis (37). In four tumors analyzed, the number of macrophages in areas with low EMAP-II immunopositivity was significantly lower than in areas of high immunopositivity (72 vs. 457 cells/mm^2).

It is assumed that endothelial adhesion molecules such as ICAM-1, VCAM-1, and P- and E-selectin play a role in the physiology of EMAP-II. Areas that expressed EMAP-II in primary uveal melanoma were usually also ICAM-1 positive (37). Another factor which may have a similar effect on the influx of macrophages, and which can also be produced by uveal melanoma cells, is vascular endothelial growth factor, type C (VEGF-C). No colocalization of macrophages and VEGF-C immunopositivity was seen (37). The conclusion of this set of experiments was that local expression of EMAP-II attracts macrophages to primary uveal melanomas. ICAM-1 was shown to be involved in tumor growth by itself. Using implants of uveal melanoma cells into the eyes of SCID mice, Niederkorn et al. showed that injection of anti-ICAM-1 antibodies markedly reduced tumor growth (41).

MACROPHAGES IN IRRADIATED UVEAL MELANOMAS

At least a population of macrophages in uveal melanoma are scavengers that diffusely infiltrate necrotic areas of the tumor (31). Consequently, they could play an important role in removal of the regressing intraocular tumor after local therapy (42).

Toivonen et al. (43) conducted a case-control study of 34 pairs of primary uveal melanoma, one having been primarily enucleated and the other irradiated with cobalt, ruthenium, and iodine plaques, and secondarily enucleated because of incomplete regression (18 eyes) or complications like recalcitrant secondary glaucoma (16 eyes). The eyes were matched on the basis of involvement of the ciliary body by the tumor, tumor height, cell type, and grade of pigmentation; the latter three factors were known to be associated with the number of infiltrating macrophages (31). Macrophages were identified with mAb PG-M1. The morphologic type and overall distribution of macrophages was similar in the two groups, except for the presence of more extensive necrotic areas with round macrophages in the irradiated tumors (43). Somewhat unexpectedly, the number of macrophages in non-necrotic areas of irradiated melanomas was approximately as often smaller than larger as compared to matched non-irradiated melanomas (43).

Because the study design was cross-sectional, and can only elucidate the situation at the time of enucleation, the results do not exclude a faster turnover of macrophages in irradiated tumors over time (43). In fact, the tumors contained apparently nonviable macrophages that were still immunopositive for the CD68 epitope. Nevertheless, it seems possible that the tumor-infiltrating macrophages do not necessarily notably increase in number in irradiated melanomas, except in areas of necrosis.

PRESENCE OF MACROPHAGES IN METASTATIC UVEAL MELANOMA

Toivonen et al. (44) also compared macrophage infiltration in primary uveal melanomas and metastases from the same tumors. They did not observe a large difference in the number of macrophages in melanomas that metastasized as compared to their hepatic metastases, but the power of the study to detect small to moderate differences was low.

CONCLUSION: THE DUAL ROLE OF MACROPHAGES IN CANCER

If macrophages are attracted to certain tumors, including primary uveal melanomas, why do they not subsequently get rid of the tumor by effectively stimulating the immune system? As shown in the animal model, sensitization against a tumor may not necessarily lead to killing of that tumor (33,34). Also, while macrophages may be involved in presenting antigens to the local or systemic immune system, they have other roles as well (45).

Macrophages that have been stimulated by interleukin-2, interferon, and interleukin-12 may become cytotoxic and kill surrounding tumor cells; on the other hand, following exposure to TGF-β, macrophages may have immune-inhibitory capacities (46,47). Because ocular fluids contain high concentrations of TGF-β, intraocular macrophages may already be biased towards an immunosuppressive function, but this has not yet been proven.

Tumor-infiltrating macrophages are also a source of a wide variety of growth factors and cytokines, which locally affect vascular endothelial and mesenchymal cells; e.g., they can induce angiogenesis, which would be beneficial for the tumor (45). It also has been postulated that macrophages may drill holes in the extracellular matrix and create pathways for new vessels, thereby assisting and promoting tumor growth (48).

These examples demonstrate the dual potential of macrophages: on the one hand, they are considered important in the removal of tumor cells; on the other hand, they may stimulate tumor growth. Naturally, it is also possible that macrophages in primary uveal melanomas act mainly as scavengers, particularly in rapidly growing tumors with a high cell turnover rate (31). In that case, the presence of tumor-associated macrophages would just be a sign of an "ugly" tumor, similar to the findings regarding tumor-infiltrating lymphocytes (49). Clearly, the role of tumor-infiltrating macrophages in uveal melanoma is complex, and will remain a highly interesting area for research for years to come.

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Index

ACAID. *See* Anterior chamber-associated immune deviation (ACAID)
Acinar cells
  lacrimal glands
    pathways, 95
AD. *See* Atopic dermatitis (AD)
Adaptive immune responses
  DC, 132–133
Aeroallergens, 186
Age-related macular degeneration (AMD), 209
Age-specific T cells, 59
Allergens
  conjunctival mast cells, 186
  LC, 184
  mite, 186
Allergic keratoconjunctivitis, 185
Allergic rhinitis, 75
Allergy
  skin vs. ocular, 183–187
AMD. *See* Age-related macular degeneration (AMD)
Amyloid P, 210
Anterior chamber-associated immune deviation (ACAID), 19, 203–205
  APC, 21
  cellular interactions, 20, CP1
  chemokines role, 22
  eye immune responses, 76
  systemic nature, 122
Antigen presenting cell (APC). *See also* Cornea, APC
  ACAID, 21
  activation of, 79–80
  anterior segment of eye, 76–79
  blood-ocular barriers, 64
  cell interaction, 17–23
  classification of, 72–75
  cross-linking of, 79–80
  cytokines, 21–22
  DC, 17, 27
  definition, 45
  distribution of, 71–81, 76
  donor purging, 161
  DTH response, 125
  effector sites, 101–103
  eye-derived, 46, 121
    antigens, 127
    bacterial and parasitic infections, 169–177
    characterization of, 123–124
    distribution, 45–64
    glaucoma pigmentary forms, 199–206
    molecular mechanisms, 124–127
    therapeutic manipulation in corneal transplantation, 157–162
    tolerance characteristics, 20–21
  host
    indirect pathway of alloimmunization, 160
    preventing corneal graft rejection, 161–162

[Antigen presenting cell]
immune deviation, 124, 127
immune response, 15, 45, 46–47, 81, 127
immunogenic, 22
MG activation, 62
microarray analysis, 124
molecules, 21–22
Mphs, 27
MZ, 18
phenotype of, 71–81
preventing corneal allograft rejection, 160
recruitment into cornea, 151–152, 153
regulatory cells, 19–21
retina, 59–63
RNA interference strategy, 127
therapeutic strategies, 81
tolerogenic, 22
transcription factor, 126
treatment of, 102
Antigen receptors
signaling by, 39
Antigen tyrosinase-related protein 2, 41
Antigen uptake
DC, 38–39
APC. *See* Antigen presenting cell (APC)
Apoptosis.
direct induction, 40
Apoptotic T cells
IL-10, 194–195
Aqueous humor (AqH), 200
Asthma, 75
Atopic dermatitis (AD), 75, 183
Atopic diathesis, 183
Autoallergens
AD, 185

Bacillus laterosporus, 140
Bone marrow (BM)
derived DC, 133, 136
culture conditions, 137
cytotoxic responses, 141
murine, 141
transplantation, 136
Boyden chamber assay
LC, 173
Bruch's membrane, 209, 211

C-type lectins
pathways of, 39–40
Calcitonin gene-related peptide (CGRP)
cornea, 172–173
Camero-splenic axis, 59
Carbohydrate recognition domains (CRDs), 39
Cell migration
induction of immunity, 27–32
maintenance of tolerance, 27–32
Cellular proteins
proteasome, 11
Central cornea
DC, 50–51
immune responses, 49–51
Central nervous system (CNS)
lymphocyte patrol, 60
CGRP. *See* Calcitonin gene-related peptide (CGRP)
Chemokines
ACAID, 22
macrophage-derived, 106
Choriocapillaris, 213
Ciliary body
Mphs, 54–55
Ciliary body stroma, 77
Ciliary processes
DC, 57
immunoperoxidase staining of, 55
CLP. *See* Common lymphoid progenitors (CLP)
CNS. *See* Central nervous system (CNS)
Collaborative Ocular Melanoma Study, 220
Common lymphoid progenitors (CLP), 1
Conjunctiva ducts
defense mechanisms, 83–85
Conjunctival epithelial cell damage, 187
Conjunctival mast cells
allergen stimulation, 186
Cornea
allografts
immune privilege, 158
immunogenicity, 161
preventing rejection, 160

[Cornea]
APC, 151–152, 153
functional aspects, 152–153
heterogeneous population, 152
HSK, 165–167
lymphoid tissues, 153–154
transplantation, 151–154
central
DC, 50–51
immune responses, 49–51
DC, 172–173
LC, 158, 170–171
limbus
confocal images, 52
epifluorescence microscopy, 49
fluorescent antigen injection, 54
immune responses, 48–53
neuropeptides, 172–173
RTMs distribution, 51
SP, 172–713
CRD. *See* Carbohydrate recognition domains (CRDs)
CsA. *See* Cyclosporine A (CsA)
CTL. *See* Cytotoxic T lymphocyte (CTL)
Cyclosporine A (CsA), 137–138
TNF, 138
Cytokines
APC interactions, 21–22
Cytoplasmic proteins
TGN, 96
Cytotoxic T lymphocyte (CTL), 159

DBA/2J
AqH, 202–203
blood: ocular barrier, 202–203
causation vs. coincidence, 203–204
DC, 203–204
glaucoma, 203–204
immune status, 202–203
immunosuppressive microenvironment, 202
iris disease
APC, 201–202
clinical presentation, 200, CP4
DC. *See* Dendritic cells (DC)
DCIR. *See* Dendritic cell immunoreceptor (DCIR)
Delayed type hypersensitivity (DTH), 122, 159
APC, 125
cornea, 171
Dendrite morphology
immature DCs, 46
Dendritic cell immunoreceptor (DCIR), 39
Dendritic cells (DC), 151, 169–170
activation, 101
status influences, 40–41
adaptive immune responses, 132–133
antibody-mediated targeting, 41
antigen uptake, 38–39
APC, 17, 27
BM-derived, 133
culture conditions, 137
cytotoxic responses, 141
cell development, 1
central cornea, 50–51
ciliary processes, 57
classification of, 72
confocal analysis, 78
cornea, 172–173
CsA
administration, 138
inhibitory effects, 138
DBA/2J, 203–204
iris disease, 201–202
definition, 131
development, 3, 135
distribution, 53
genetic engineering of, 141
graft acceptance in hosts, 50
HLA, 9
homing directions, 28
HSV-1, 166
immune response role, 63
immunity induction, 41
induction of tolerance, 40–41
infection, 176
inflammatory conditions, 32
iris pigment epithelium, 77
isolation, 133–134
L. donovani, 176
LC, 29, 71
LPS, 166–167
maturation expressions, 32

[Dendritic cells (DC)]
  MHC, 37
    gene products, 132
  migration, 31
    maturation, 31–32
    mechanisms, 30–31
  monocyte-derived, 29–30
  monocyte differences, 27–28
  morphology, 53
  mouse eyelid, CP2
  Mphs differences, 27–28
  ocular surface system, CP3
  organ transplantation, 134
  parasitic infection, 175
  peripheral tolerance, 131
  Peyer's patch, 135
  phagocytosis, 38
  pharmacologically manipulated, 137–140
  phenotype, 53
  pinocytosis, 38
  polarizing mechanisms, 74
  prolactin, 105
  Pseudomonas aeruginosa, 176
  Pseudomonas aeruginosa keratitis, 173–174
  regulatory, 134
  S-antigens, 191
  signaling milieus, 101
  specialized lineage model, 2
  specifically cultured, 136–137
  spontaneous migration, 32
  subtyping of, 72
  T cells, 42, 80, 106
    proliferation, 42
  TLR binding, 15
  tolerance, 102
    versus immunity, 37–42
    induction, 133–137, 142
  tolerogenic properties, 142
  transport vehicle, 38
  uveal tract of eye, 56–58
Deoxyspergualin (DSG), 140–141
Dermatitis, atopic, 75, 183
Dexamethasone, 139–140
Diathesis
  atopic, 183
Dimeric immunoglobulin A (dIgA), 95
Direct pathway of alloactivation, 159
Donor antigen presenting cell
  purging, 161
Donor Langerhans cells
  orthotopic corneal graft rejection in rodents, 160
Drusen
  HLA-DR, 210–211
  PNA, 210–211
Dry eye, 88
  eye-associated lymphoid tissue, 83–89
DSG. *See* Deoxyspergualin (DSG)
DTH. *See* Delayed type hypersensitivity (DTH)
Dust, 186

EAE. *See* Experimental autoimmune encephalomyelitis (EAE)
EALT. *See* Eye-associated lymphoid tissue (EALT)
EAU. *See* Experimental autoimmune uveoretinitis (EAU)
ECP. *See* Eosinophilic cationic proteins (ECP)
Effector sites
  signaling, 99–101
EMAP. *See* Endothelial monocyte-activating polypeptide (EMAP)
Encephalomyelitis
  experimental autoimmune, 133
Endocytosis, 73
Endogenous posterior uveitis (EPU), 190
Endoplasmic reticulum (ER), 10
Endothelial monocyte-activating polypeptide (EMAP), 221–222
Eosinophilic cationic proteins (ECP), 185
Epidermal effector cells
  hyper-reactivity, 183
Epithelial cells
  conjunctival, 187
  inflammatory, 184–185
  lacrimal secretory
    functional design, 95–98
    immune functions, 94
  transepithelial transport, 84
EPU. *See* Endogenous posterior uveitis (EPU)

ER. *See* Endoplasmic reticulum (ER)
Experimental autoimmune encephalomyelitis (EAE), 133
Experimental autoimmune uveoretinitis (EAU), 190
Eye(s)
  allergy, 185–186
    IgE, 185
    vs. skin allergy, 183–187
  antigens
    APC, 127
    immune response to, 122
  APC, 45–64, 46, 121
    antigens, 127
  immune, 121
    ACAID, 76
  Mphs populations, 63–64
  surface system
    APC, 93–108
    DC, 104
    fluid-secreting functions, 94
    immunoregulation, 104–108
    infection, 94
    lymphocyte cycles, 93–108
    mucosal immune functions, 94
  tolerance
    APC, 20–21
    immune system, 132
Eye-associated lymphoid tissue (EALT), 84
  development, 89
  entrance site, 85–89
Eye-derived antigen presenting cell, 46, 122–123
  bacterial and parasitic infections, 169–177
  characterization of, 123–124
  distribution, 45–64
  glaucoma pigmentary forms, 199–206
  molecular mechanisms, 124–127
  therapeutic manipulation in corneal transplantation, 157–162
  tolerance characteristics, 20–21
Eyelid
  cell localization, 79
  cell type identification, 77
  DC, CP2
Fas ligand (Fas-L)
  keratinocytes, 184
Fas$^+$T cells
  EAU, 194

Gastrointestinal-associated lymphoid tissue (GALT), 86
Glaucoma
  causation vs. coincidence, 203–204
  DBA/2J, 203–204
    mouse model, 200–201
  immune abnormalities, 201–202
  pigmentary, 200
Glucocorticoid, 139
Glycoprotein B, HSK, 167
Glycoprotein (transmembrane) nmb (Gpnmb), 201
GM-CSF. *See* Granulocyte-macrophage-colony stimulating factor (GM-CSF)
GMP. *See* Granulocyte/macrophage progenitors (GMP)
Gpnmb. *See* Glycoprotein (transmembrane) nmb (Gpnmb)
Graft-versus-host-disease (GVHD), 136
Granulocyte-macrophage-colony stimulating factor (GM-CSF), 136
  keratinocytes, 184
Granulocyte/macrophage progenitors (GMP), 1
Grass pollen, 186
GVHD. *See* Graft-versus-host-disease (GVHD)

Hematopoietic cell lineage, 3
Hematopoietic progenitor cells (HPCs), 71
Hematopoietic stem cells (HSC), 1
  long-term, 1
Herpes simplex keratitis (HSK), 165–167
  corneal APC, 165–167, 166
Herpes simplex virus 1 (HSV-1)
  DC, 166
HLA. *See* Human leukocyte antigen (HLA)
Host antigen presenting cell
  indirect pathway of alloimmunization, 160
  preventing corneal graft rejection, 161–162

House dust, 186
HPC. *See* Hematopoietic progenitor cells (HPCs)
HSC. *See* Hematopoietic stem cells (HSC)
HSK. *See* Herpes simplex keratitis (HSK)
HSV. *See* Herpes simplex virus 1 (HSV-1)
Human leukocyte antigen (HLA), 9–16
  antigen pathways, 11–12
  antigen presentation, 10
  binding protein chain, 13
  class I, 10
  class II, 10–11
  DC, 9
  DR antibodies, 11, 210, 211
    choroid, 212, 213
    core domains, CP4
    drusen, 211, 214
    immunoreactivity, 212
    sclera, 212
  MHC, 12
  peptide
    presentation, 12
    selection, 14
  polymorphism, 13–14
  specificity, 12–13
Hyperbaric oxygen
  donor LC, 161

ICAM-1. *See* Intracellular adhesion molecule-1 (ICAM-1)
IDEC. *See* Inflammatory dendritic epidermal cells (IDEC)
IFN. *See* Interferon-gamma (IFN-gamma)
IgE. *See* Immunoglobulin E (IgE) receptors
IL. *See* Interleukin-1 (IL-1)
Immune deviation, 86. *See* also ACAID
  APC, 127
  induction, 121–127
Immune privelage, 151
Immunoglobulin E (IgE) receptors, 75–76
Immunoreceptor tyrosine-based activation motifs (ITAM), 75
Immunoreceptor tyrosine-based inhibition motif (ITIM), 39
Infectious tolerance, 102
Inflammatory dendritic epidermal cells (IDEC), 184
  AD, 185
Inflammatory stress
  GM-CSF, 3
Interferon-gamma (IFN-gamma)
  AD, 185
  cornea, 171
  HSK, 165
Interferon-producing cell development (IPC), 1–4
  developmental capacity, 3
Interleukin-1 (IL-1)
  APC recruitment into cornea, 152
  LC, 161
Interleukin-4 (IL-4)
  cornea, 171
Interleukin-6 (IL-6)
  retinal pigment epithelial cells, 195
Interleukin-8 (IL-8)
  keratinocytes, 184
  retinal pigment epithelial cells, 195
Interleukin-10 (IL-10)
  cornea, 171
Interleukin-15 (IL-15)
  retinal pigment epithelial cells, 195
Interphotoreceptor retinoid binding protein (IRBP), 190
Intracellular adhesion molecule-1 (ICAM-1), 152, 222
  keratinocytes, 184
Intracellular membrane traffic
  self-tolerance, 97
IPC. *See* Interferon-producing cell development (IPC)
IRBP. *See* Interphotoreceptor retinoid binding protein (IRBP)
Iris
  DBA/2J
    APC, 201–202
    clinical presentation, 200, CP4
  DC, 77
  Mphs, 54–55
  pigment epithelium, 77
Irradiated uveal melanomas
  macrophages, 222
ITAM. *See* Immunoreceptor tyrosine-based activation motifs (ITAM)
ITIM. *See* Immunoreceptor tyrosine-based inhibition motif (ITIM)

Keratinocytes
  ICAM-1, 184
Keratitis
  herpes simplex, 165–167
    corneal APC, 165–167
  Pseudomonas aeruginosa, 173–174
Keratoconjunctivitis, allergic, 185

Lacrimal epithelium
  ocular surface system PAC and lymphocytes, 93–108
Lacrimal glands
  acinar cell pathways, 95
  immunomodulatory paracrine mediators, 107
  prolactin, 105
  systemic factors influence, 105
Lacrimal secretory epithelial cells
  autoantigen exposure, 95–98
  functional design, 95–98
  immune functions, 94
Lacrimal-ocular surface system (LOS), 83
Langerhans cells (LC), 2, 151
  allergen uptake, 184
  cornea, 170–171
    graft, 159
    grafts, 158
  DC, 29, 71
  donor, 160
  emigration molecular processes, 30
  graft acceptance in hosts, 50
  orthotopic corneal graft rejection in rodents, 160
  surface density of, 50
Langerin, 72
LC. *See* Langerhans cells (LC)
LDOT. *See* Low dose oral tolerance (LDOT)
Leishmania
  IL-12, 175
Leishmania donovani
  DC, 176
Limbus
  immune responses, 51–52
Lipopolysaccharide (LPS), 136
  DC, 166–167
Long-term hematopoietic stem cells, 1
LOS. *See* Lacrimal-ocular surface system (LOS)
Low dose oral tolerance (LDOT)
  ACAID, 19
LPS. *See* Lipopolysaccharide (LPS)
Lymph node
  soluble antigen transport, 28–29
Lymphocytes
  ocular surface system, CP3
  uveal melanoma, 219

M cell
  antigen uptake, 87
Macrophage-derived chemokines (MDC), 106
  keratinocytes, 184
Macrophages (Mphs), 27, 151. *See also* Resident tissue macrophages (RTMs)
  acquired deactivation, 48
  alternative activation, 47–48
  central cornea, 50–51
  choroid, 54–55
  ciliary body, 54–55
  classical activation, 47
  dual cancer role, 223
  homing directions, 28
  humoral activation, 47
  immune response role, 63–64
  immune responses, 47–48
  innate activation, 47
  iris, 54–55
  irradiated uveal melanomas, 222
  uveal melanoma, 217–223
    determination, 221
    tumor immunology, 220–221
Macropinocytosis, 73
Major basic protein (MBP), 185
Major histocompatibility complex (MHC). *See* also HLA
  class densities, 59
  class II antigens, 158, 159, 166, 169
    DC, 37
    drusen, 209–214
    gene products, 132
    keratinocytes, 184
  peptide TCR, 190
MALT. *See* Mucosa-associated lymphoid tissue (MALT)

Mammary glands
systemic hormonal influences, 100
Marginal zone (MZ)
APC, 18
Mast cells
conjunctival allergen stimulation, 186
hyper-reactivity, 183
ocular allergy, 186
MBP. *See* Major basic protein (MBP); Myelin-basic protein (MBP)
MDC. *See* Macrophage-derived chemokines (MDC)
Megakaryocyte/erythrocyte progenitors (MEP), 1
Melanoma. *See* Uveal melanoma
MEP. *See* Megakaryocyte/erythrocyte progenitors (MEP)
Meshwork (MW), trabecular, 58
Metastases, 223
Metastatic uveal melanoma
macrophages, 223
MG. *See* Microglia (MG)
MHC. *See* Major histocompatibility complex (MHC)
Microglia (MG)
advances in, 60
APC, 60, 62
dendriform cells, 61
immune responses, 60–63
ramified cells, 61
retina, 60–63
MIP-2, 175
Mite allergens, 186
Mixed leukocyte reaction (MLR), 135
MMF. *See* Mycophenolate mofetil (MMF)
Mouse eyelid
DC, CP2
Mphs. *See* Macrophages (Mphs)
MPP. *See* Multi-potent progenitors (MPPs)
Mucosa-associated lymphoid tissue (MALT), 84
activation of, 88
antigen responses, 89
antigens, 98
definition, 87
[Mucosa-associated lymphoid tissue (MALT)]
function of, 85
lymph nodes, 98
organization of, 88
Mucosal immune system
APC initiation, 98–99
default response, 103
immature dendritic cells, 101
systemic hormones, 100
Multi-potent progenitors (MPPs), 1
Murine bone marrow derived dendritic cells
cytotoxic responses, 141
MW. *See* Meshwork (MW), trabecular
Mycophenolate mofetil (MMF), 140
Myelin-basic protein (MBP), 133
MZ. *See* Marginal zone (MZ)

Nasal-associated lymphoid tissue (NALT), 86
Nasolacrimal ducts
defense mechanisms, 83–85
Natural killer (NK) cells, 18
direct induction, 40
IL-10, 194–195
uveal melanoma, 219
Neuraminidase, 210
Neuropeptides
cornea, 172–173
Nitric oxide (NO)
retinal pigment epithelial cells, 195
NK. *See* Natural killer (NK) cells
NLS. *See* Nuclear localization signal (NLS)
NO. *See* Nitric oxide (NO)
Nuclear localization signal (NLS)
protein binding, 126

Ovalbumin (OVA), 123
delayed hypersensitivity, 203

Parasitic infection
DC, 175
Parkinson's disease, 62
Passenger cells, 159
PDS. *See* Pigment dispersion syndrome (PDS)

Peanut agglutinin (PNA), 210, 211
  drusen core domains, CP4
PEC. *See* Peritoneal exudate cells (PECs)
PECAM. *See* Platelet endothelial cell adhesion molecule (PECAM)
Penicillium, 140
Peritoneal exudate cells (PECs), 123
Perivascular macrophages, 57
Peyer's patches, 102
  DC, 135
PG. *See* Pigmentary glaucoma (PG)
Phagocytosis, DC, 38
Pigment dispersion syndrome (PDS), 200
Pigmentary glaucoma (PG), 200
  DBA/2J mouse model, 200–201
  immune abnormalities, 201–202
Pituitary, prolactin
  production patterns, 108
Plasma differentiation, 99
Platelet endothelial cell adhesion molecule (PECAM), 175
PNA. *See* Peanut agglutinin (PNA)
Polarized cells, 74
Programed cell death, 63
Prolactin, 100
  DC, 105
  lacrimal glands, 105
  production patterns, 108
  spleen, 105
Protein tyrosine kinase (PTK), 80
Pseudomonas aeruginosa, 170–171
  cornea, 171–172
  DC, 176
  keratitis, 173–174
  LC/B7/CD28 costimulatory pathway, 171–172
PTK. *See* Protein tyrosine kinase (PTK)

Ragweed, 186
Rapamycin (RAPA), 139
  endocytosis, 139
Regulatory dendritic cells
  review articles, 134
  tolerance induction, 131–142
Resident tissue macrophages (RTMs), 47
  distribution, 53
  morphology, 53
  phenotype, 53
Retina
  APC, 59–63
  autoantigens, 190
  lacrimal glands, 93
  microglia immune responses, 60–63
  ocular surface tissue, 93
Retinal pigment epithelium (RPE), 209
Rhinitis, allergic, 75
RNA interference strategy, APC, 127
RPE. *See* Retinal pigment epithelium (RPE)
RTM. *See* Resident tissue macrophages (RTMs)

S-antigens, 190–191
  DC, 191
Sirolimus, 139
Sjögren's syndrome, 94, 107
Skin allergy vs. ocular allergy, 183–187
Sodium antimony gluconate
  L. donovani, 176
SP. *See* Substance P (SP)
Spleen, prolactin, 105
Staphylococcus aureus
  AD, 185
  ocular allergy, 185
Stress
  inflammatory GM-CSF, 3
Submandibular lymph node, 87
Substance P (SP)
  cornea, 172–173

T cell
  activation requirements, 15, 88
  age-specific, 59
  allergic responses, 74
  anergy induction mechanism, 134
  APC, 80
  apoptotic, 194–195
  cornea, 172
  DC, 42, 80
  IL-10, 194–195

[T cell]
LCS, 80
MHC molecules, 17
MHC peptide complexes, 37
proliferation, 42
Pseudomonas aeruginosa, 172
regulatory types, 42
T cell receptor (TCR)
genes, 192
ovalbumin, 132
TALT. *See* Tear duct-associated lymphoid tissue (TALT)
TAP. *See* Transporter antigen processing (TAP)
TARC. *See* Thymus activation regulated chemokine (TARC)
Tear duct-associated lymphoid tissue (TALT), 84
dry eye, 88
TGN. *See* Trans-Golgi network (TGN)
Thrombospondin (TSP), 124–125
APC, 127
extracellular matrix protein, 125
Thymic-stromal lymphopoetin (TSLP)
keratinocytes, 184
Thymus activation regulated chemokine (TARC), 106
keratinocytes, 184
TLR. *See* Toll-like receptors (TLR)
TNF. *See* Tumor necrosis factor (TNF)
Tolerogenic antigen presenting cell, 22
Toll-like receptors (TLR), 170
DC, 15
future, 176–177
Pseudomonas aeruginosa keratitis, 173–174
Toxoplasma
IL-12, 175
Trabecular cells
outflow pathways, 58–59
phagocytic properties, 58
Trabecular meshwork (MW), 58
Trans-Golgi network (TGN), 96
immature secretory vesicles, 97
transport vesicles, 96
Transcytosis, 96
Transepidermal water loss, 183
Transporter antigen processing (TAP), 10
TRP. *See* Tyrosinase-related protein 1
TSLP. *See* Thymic-stromal lymphopoetin (TSLP)
TSP. *See* Thrombospondin (TSP)
Tumor necrosis factor (TNF)
CsA, 138
Tumor necrosis factor-alpha (TNF-alpha), 152
keratinocytes, 184
retinal pigment epithelial cells, 195
Tyrosinase-related protein 1, 201
Tyrosinase-related protein 2, 41

Ultraviolet (UV) irradiation
donor LC, 161
Uveal melanoma
HLA-A, 218–219
HLA-B, 218–219
intraocular tumor, 220–221
irradiated macrophages, 222
lymphocytes, 219
macrophages, 217–223, 219–220
determination, 221
tumor immunology, 220–221
metastatic, 223
NK cells, 219
prognostic factors, 218–219
systemic immunization animal model, 220–221
Uveal tract
DC, 56–58
immune responses, 53–58
Uveitis
antigen specificity, 194
APC, 189–195
endogenous posterior, 190
vitreous cells, 193
immune processes, 192
immune responses regulation, 193–194
vitreous T cells, 194
Uveoretinitis
experimental autoimmune, 190

Vascular cell adhesion molecule-1 (VCAM-1), 222

Vascular endothelial growth factor receptor (VEGFR), 153
Vasoactive intestinal peptide (VIP), 99
  cornea, 172–173
VCAM-1. *See* Vascular cell adhesion molecule-1 (VCAM-1)
VEGFR. *See* Vascular endothelial growth factor receptor (VEGFR)
Veiled cells, 31
VIP. *See* Vasoactive intestinal peptide (VIP)

Xerosis, 183

Ophthalmology

about the book...

In recent years the importance of antigen-presenting cells and their influence on the eyes has been elucidated. We have learned that nearly every structure of the eye harbors these cells, yet they differ in their activities and thus in the ways in which they protect against — or participate in — diseases of the eye. ***Antigen-Presenting Cells and the Eye*** summarizes current knowledge about ACPs in general, and their role in the eye, in particular.

Key features within ***Antigen-Presenting Cells and the Eye*** include information about the development, differentiation, and migration of antigen-presenting cells…details the role of antigen-presenting cells as mediators of specific and unspecific immune system responses…explains how antigen-presenting cells respond in the presence of infectious diseases (viral, bacterial, and parasitic), as well as in inflammatory disorders of the external segment and other important ocular disorders…suggests how future therapies will target antigen-presenting cells to reduce immune tolerance in the eye

about the editors...

MANFRED ZIERHUT is Associate Professor of Ophthalmology, University Eye Hospital, Tübingen, Germany. Dr. Zierhut received his M.D. and Ph.D. from University of Hannover, Germany, and has published more then 100 articles, written or co-edited 30 books, and completed over 3000 surgeries in ophthalmology.

HANS-GEORG RAMMENSEE is Department Head of Immunology, University of Tübingen, Germany, Director of the Interfacultary Institute for Cell Biology, and Chairman of the *Graduiertenkolleg Cellular Mechanisms of Immune-associated Processes*, German Research Council. In conjunction with his academic assignments, Dr. Rammensee sits on the editorial boards of *Immunogenetics* and *Current Immunology Reviews*, he received his Ph.D. in Biology, University of Tübingen, Germany, and is a member of the Evaluation Committee for the Cooperation in Cancer Research between the German Cancer Research Center and the Israel Ministry of Science.

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