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Joan Stein-Streilein *Editor*

Infection, Immune Homeostasis and Immune Privilege

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Preface

This book is aimed at reviewing the immune homeostatic mechanisms that regulate inflammation in the context of the innate and the adaptive immune response, the microbiome, and the environment at the level of the organ/tissue and in relation to the select infections that invade those organs.

A powerful protector of mammalian organs and tissues is an omniscient and plastic immune response. The immune response begins with innate responses that are able to respond immediately to infectious agents or foreign substances. These are induced by cells of myeloid lineage as well as select lymphocytes. The adaptive immune response results from sophisticated processes that sense antigens in the periphery and transport them to the secondary lymphoid organs where cells are poised in G_0 ready to differentiate into a variety of immune effector cells that deal with the antigenic threat both locally and peripherally. Similarly, adaptive immune responses are affected by cells of the myeloid and lymphoid lineage. Adaptive immune responses are associated with cytokines and antibodies that induce the accompanying inflammation. Both the innate and the adaptive immune responses induce inflammation that has the potential of leading to tissue damage. In order to prevent unregulated inflammation that causes tissue destruction and disease, self-imposed regulatory mechanisms are triggered almost simultaneously with the induction of the response to ensure its safe termination and restore immune homeostasis in the organ/tissue. The initiation of the immune response is dependent on secondary lymphoid tissue for the primary response to occur, but the immune regulation of the innate and the adaptive immune response occurs at the level of the tissue. Therefore, the regulatory response is fashioned to preserve the function of that tissue by limiting inflammation.

This book seeks to connect the knowledge of immune regulation in one tissue with another. In the eye the tissue is highly protected from inflammation and is left to defeat infectious invaders with mechanisms that lack inflammation. The eye, brain, reproductive tract, and (more recently) the liver are tissues that are commonly accepted to express immune privilege. The lung and gut, however, share many of the mechanisms of immune privilege as the reader will discover when reading these chapters. The lung and gut both have high levels of TGFβ and

importantly use mechanisms to suppress the antigen-presenting ability of the local dendritic cell/macrophage populations.

Much of our information on immune privilege comes from the eye. In order to protect the visual pathway, highly sophisticated mechanisms cooperated to limit damaging inflammation from occurring. Hazlett's and Stein-Streilein's chapter on the eye focuses on the innate and the adaptive immune regulatory mechanisms in detail and considers the infections that occur mostly in the anterior portion of the eye, as well as how they are treated.

The testes and the placental, both considered to be immune privileged sites, have developed similar and unique mechanisms to those we know that exist in the eye. Mark Hedger has very effectively completed his task of clarifying the meaning, mechanisms, and manifestations of immune privilege in the testes with academic grace. Like the eye, although inflammation in the testis is controlled by multiple overlapping mechanisms of immune suppression, the testes do not display an increased susceptibility to tumors or infections compared to other tissues and are actually rare compared to more distal tissues of the male reproductive tract. Nagamatsu and Schust focused their review on how the immune privilege in the placenta allows the genetically disparate fetus to thrive and grow without immune attack by the allogeneic maternal cells. They explain how gestational hormones and placenta-derived substance actively modulate maternal immunity and promote tolerance to the antigenically disparate fetus. Their well-conceived review considers the costs of immune privilege in the placenta in terms of increased infectious disease in mother and fetus.

The liver is a tissue that is unique in its ability to regenerate and is the only tissue that can be successfully transplanted across allogeneic lines without the need for immunosuppression. Not only is it accepted, but if a tissue that is normally rejected is cotransplanted with the liver, it too is protected. Wohlleber's and Knolle's review delightfully educates us on the unique qualities of the immune privilege in the liver.

Chang, DeKruyff, and Umetsu have carefully focused their review on immune homeostasis in the lungs in the regulation of asthma. Their presentation adds insight into the readers' understanding of the "Hygiene Hypothesis" and stimulates new thoughts that connect the ideas presented about the gut and microbiome to the lung.

Last, but ever so important, the bacteria in the gut are controlled by compartmentalization, monitoring, and selection of the microbial ecosystem. Some bacteria promote tolerance while others promote inflammation. Thus the prevention of harmful inflammatory responses is the result of a joint effort on the part of the host's immune system, the physiology of the host, and the members of the gut microbial community (microbiome) and can be directly influenced by the environment. Wroblewska and Nagler have elegantly simplified the complicated interactions and mechanisms of the homeostasis and privileged protection of the gut from food antigens and bacteria in their chapter.

Overall, we might conclude that if left unregulated, the inflammatory response can be the most destructive aspect of the immune response. Inflammation is the offense for both the innate and the adaptive immune response. Unlike the adaptive immune response, cells of the innate immune response are not dependent on

secondary lymphoid organs for their generation and function. Innate cells are able to respond immediately to danger, in part by their expression of receptors that recognize pathogen-associated molecular pattern (PAMP). Such receptors include Toll-like receptors, adhesion molecules, and scavenger molecules. Activation of PAMP receptors induces the production and release of inflammatory cytokines and complement factors. The adaptive immune response requires that the lymphocytes differentiate into effector cells and only after their differentiation they are capable of secreting inflammatory cytokines and contributing to tissue destruction. However, built into the immune response is the simultaneous production of suppressive molecules, the downregulation of activating receptors, the inhibitors of their signaling pathways, and the molecules that when linked to their ligands will induce apoptosis.

Within the last decade, the importance of nonlymphoid cells in immune regulation and the immune homeostasis of tissues has become known. Molecules that were thought to be markers of lymphocytes now are known to be expressed by endothelial, epithelial, and various other stromal cells. These molecules and receptors thus allow the stromal cells to interact and regulate the lymphocytes within their environment. And yes, we continue to learn.

Boston, MA Joan Stein-Streilein

Contents

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The Eye as a Model for Immune Privilege

Linda D. Hazlett and Joan Stein-Streilein

Abstract Immune privilege was first observed in the eye as early as 1873 when tumors, and later skin allografts were accepted for extended periods of time in the eye. Immune privilege allows for protection against infectious organisms in the absence of inflammation. This chapter organizes our current knowledge of immune privilege and immune homeostasis of the eye in relation to health and infectious disease. This review discusses the role of innate and adaptive immune regulation in relation to the ocular environment and the immunosuppressive molecules on both immune and stromal cells. Microorganisms that cause bacterial keratitis are listed and current therapeutical approaches are discussed.

Keywords Anterior chamber • Aqueous humor • Immune privilege • Neuropeptides • *Pseudomonas aeruginosa* • *Staphylococcus aureus*

1 Introduction

The eye is the gateway for sending environmental information to the brain (Fig. 1). It sends images to the brain for interpretation via the retina that perceives information only within a narrow band of light. Seventy percent of the information received by the brain is through the eye. The critical functions of the eye are protected by highly sophisticated anatomical, biochemical, and immunological mechanisms that help to prevent destructive forces from gaining access to this window for the brain.

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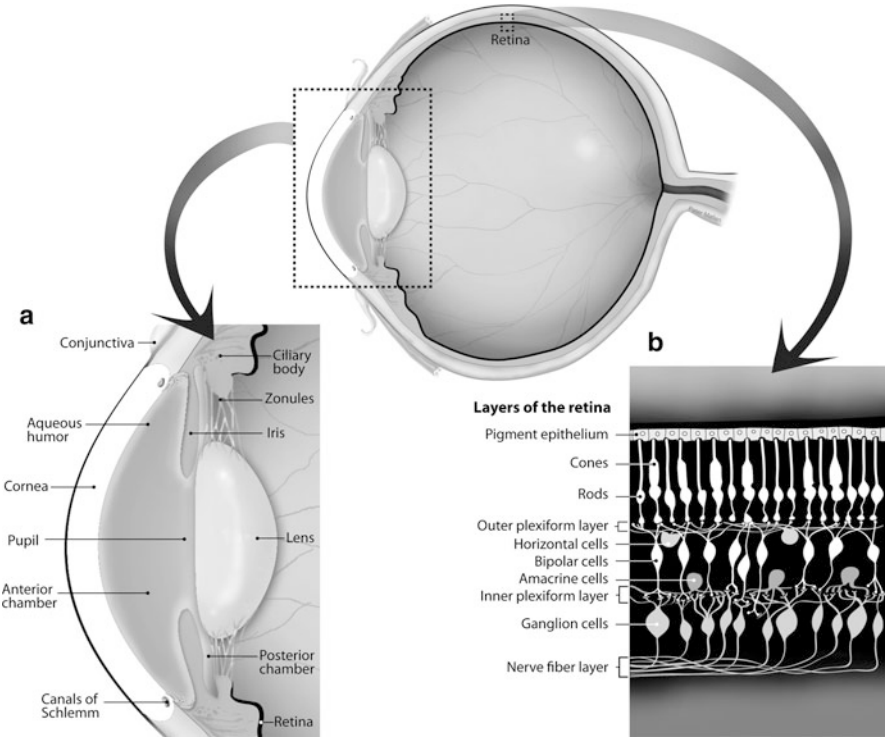

Fig. 1 Illustration of the eye. (**a**) Enlarged view of the anterior chamber showing detailed anatomy. (**b**) Enlarged view of the retina showing the various layers of the cells from the outside into the vitreous cavity

Multiple regulatory mechanisms contribute to a highly orchestrated security system called immune privilege that is evident in other organs that are critical to life, including the reproductive tract and the brain itself.

Immune privilege was first observed in the eye when tumors and later skin allografts were accepted for extended periods of time in the eye (van Dooremaal 1873; Medawar 1945). Immune privilege allows for protection against infectious organisms in the absence of inflammation. The privileged immune response in the eye is accomplished by both locally produced mechanisms that limit inflammatory immune responses to occur and by unique adaptive immune mechanisms in the periphery. At least three types of mechanisms are associated for the maintenance of the immune privilege and immune homeostasis of the eye:

(a) Mechanisms that allow host defense with little or no inflammation
(b) Mechanisms that block activated cells from damaging the tissue
(c) Mechanisms that induce local and peripheral tolerance to eye-associated antigens (tolerance to self-tolerance)

2 Innate and Adaptive Immune Responses

To understand how the eye is privileged, one must first understand the sequence of events that lead to innate and adaptive immune responses and immune protection. It is true that innate immune cells such as neutrophils, macrophages, NK cells, and gamma-delta cells not only contribute to host defenses against infectious organisms, but also are central to control of tumor cells and initiating adaptive immune responses that in the end contribute to the clearance of most infectious organisms. The conundrum is that one cannot have an adaptive immune response without an innate inflammatory response. Tissue macrophages not only carry the antigens/organisms to the secondary lymphoid organs for the presentation to lymphocytes for the initiation of the adaptive immune response but also promote the immediate inflammatory response. The local inflammatory response then allows peripheral adaptive immune cells and other inflammatory cells to enter the eye.

As said previously, before we have the adaptive immune cells ready to enter the eye, the innate cells within the eye must be activated. Resident F4/80-positive macrophages throughout the eye can be activated by infectious organisms or antigens released by trauma. Innate cells respond immediately to these "danger" signals (Matzinger 1994) by releasing inflammatory cytokines including TNF α, IL-1β, and IL-6. Ocular stromal cells are also capable of releasing inflammatory cytokines. Cells within the eye can release inflammatory neuropeptides, like Substance P (SP), that signal the release of inflammatory cytokines from a variety of cells. SP also is associated with inflammation outside of the eye. Inflammation allows for cells to change their form, migrate, and transport antigen to the spleen and lymph node via the vasculature and lymphatics; contributes to openings in the blood–ocular barrier; and induces increases in the expression of adhesion molecules on the vasculature that assist in the recruitment of adaptive and innate cells from the periphery.

3 Ocular (Innate) Immune Privilege: Mechanisms That Contribute to Host Defense but Lack Inflammatory Qualities

Phagocytes, including neutrophils, monocytes, and macrophages, are critical for host defense because they are the first to recognize danger signals (Matzinger 1996). A property of their defense is their killing capacity that releases tissue-destructive molecules in the process. Excessive reactive oxygen species (ROS) cause tissue destruction and contribute to chronic inflammation. Both infections and trauma can cause a hyperproduction of cytokines that has been referred to as a "cytokine storm." Thus the innate cells are highly regulated to by a complex network of inhibitory receptors that counteract and regulate the inflammatory signals. Macrophages and neutrophils express many inhibitory receptors, some of which have been examined for their function in the eye (Steevels and Meyaard 2011). For example, the ability of the eye to prevent intraocular activation of innate

Table 1 Predominant microorganisms causing bacterial keratitis

Bacteria	
Staphylococcus species	e.g., *S. aureus*
Streptococcus species	e.g., *S. pneumoniae*
Pseudomonas species	e.g., *P. aeruginosa*
Enteric Gram-negative rods	e.g., *E. coli, Klebsiella pneumoniae, Shigella flexneri*
Corynebacterium species	e.g., *C. trachomatis*
Moraxella and related species	e.g., *M. liquefaciens*

immune effectors spares the corneal endothelium from destruction by NK cells and neutrophils and protects the visual axis from distortion by macrophage and complement-mediated inflammation. However, if the system is overwhelmed by microbes, experiences trauma, or the immune privilege is compromised, then infection and inflammation will occur. Assuming the antigenic challenge is not overwhelming, the eye is protected by (1) its immunosuppressive environment, (2) regulatory molecules expressed by the ocular stromal cells, and (3) regulatory molecules on the innate cells.

In spite of its positive qualities, immune privilege in the eye precipitates a dilemma concerning how to protect against infection when inflammation, a major mediator of host defenses, is downregulated. Some aspects of this dilemma have been explored. Advances in our current understanding of host innate/adaptive immunity and the abrogation of immune privilege that occurs in ocular bacterial infections have been accomplished using experimental animal models. Two ocular bacterial pathogens that induce keratitis have been intensely studied and include *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Table 1). For *P. aeruginosa*, mouse models have shown that IL-12-driven IFNγ production in Th1 responder strains such as C57BL/6 contributes to corneal perforation, while IL-18-driven IFNγ production is associated with bacterial killing and less disease in Th2 responders (BALB/c). The role of Toll-like receptors (TLRs), neuropeptides, macrophages, regulation of neutrophil apoptosis, and defensins is also highlighted. *S. aureus* produces and secretes many proteins, including coagulase; protein A; α-, β-, γ-, and δ-toxin; and leukocidin. Animal models have been used to examine the role of these factors in keratitis and revealed the importance of several, as well as the role of PMN and TLR 2, in disease.

4 Immunosuppressive Environment of the Eye

Existence of immune regulatory molecules for the innate immune system was historically demonstrated in the anterior portion of the eye when Apte and Niederkorn provided evidence that aqueous humor (AqH) was capable of inhibiting lysis of corneal endothelial cells by NK cells (Apte et al. 1998). Since then, evidence has accumulated to indicate that factors found in normal aqueous humor (1) prevent NK cells from lysing their targets (Apte et al. 1998), (2) inhibit neutrophil activation by CD95 ligand (Ferguson and Griffith 2007; Ferguson and

Griffith 2006), (3) suppress nitric oxide production by activated macrophages (Taylor 2007), and (4) interfere with complement activation via the alternative pathway (Streilein and Stein-Streilein 2000).

Thrombospondin (TSP) is a surface molecule but also has a soluble form and has been shown to be both a stabilizer and activator of TGFβ2. TSP knockout mice will not support the development of peripheral tolerance to antigens inoculated into the anterior chamber, presumably by not being able to activate the TGFβ2 as above or like this in the environment and innate cells (Masli et al. 2002; Masli et al. 2006). TSP-1 is constitutively present in AqH, and the *TSP*-1 gene is active in ocular pigment epithelial cells and corneal endothelium (Streilein 2003) and is known for its potent angiostatic function in the eye (Streit et al. 1999; Lawler 2000; Sheibani et al. 2000). It is an immediate early gene found in antigen-presenting cells posttreatment with TGFβ2 (Masli et al. 2002).

The fluids of the eye contain a variety of neuropeptides that are immunosuppressive to both immune and innate immune cells (Taylor 2007; Stein-Streilein and Taylor 2007). Such factors include neuropeptides like vasoactive intestinal peptide (VIP) (Streilein et al. 2000; Taylor et al. 1998), somatostatin (SOM) (Taylor 2003), and calcitonin gene-related peptide (CGRP) (Taylor et al. 1998).

4.1 Transforming Growth Factor Beta (TGFβ)

While bone marrow cells can be activated to make TGFβ1, ocular stromal cells produce TGFβ2 (Cousins et al. 1991; Streilein et al. 1992). TGFβ2 is a major immunosuppressive factor in the eye. TGFβ2 has a pivotal role in the immune system to maintain tolerance, primarily by regulating lymphocyte proliferation, differentiation, and survival (Li et al. 2006; Kriegel et al. 2006). TGFβ2 inhibits the development of immunopathology to self- or non-harmful antigens without compromising immune responses to pathogens.

4.2 Alpha-Melanocyte-Stimulating Hormone (αMSH)

Another major immunosuppressive factor in aqueous humor is αMSH (Taylor et al. 1992; Taylor et al. 2006). The neuropeptide αMSH has an important role in modulating immunity and immune homeostasis. αMSH promotes the production of TGFβ and downregulates IFNγ (Taylor 2009). A recent report shows that αMSH has the ability to convert effector T cells into T-regulatory cells with antigen-specific activity (Taylor and Lee 2011). Whether this function is active in vivo has yet to be reported. Chen et al. showed that aqueous humor inhibited CD95L-dependent activation of neutrophils, and both TGFβ2 and αMSH, constituents of

aqueous humor, inhibit neutrophil-mediated killing of corneal endothelial cells (Chen et al. 1998). Furthermore, αMSH inhibits TLR4 (Toll-like receptor 4) signaling in macrophages (Zamiri et al. 2006).

4.3 Vasoactive Intestinal Peptide (VIP)

Recent studies also have provided evidence for another neuropeptide, VIP, functioning as a potent endogenous anti-inflammatory molecule affecting the immune response antithetically when compared to SP (Szliter et al. 2007). VIP regulates inflammatory mediators through several transduction pathways and transcription factors essential for gene activation, such as NF-κB, interferon regulatory factor-1 (IRF-1), mitogen-activated protein kinase (MAPK), and cAMP response element (CRE) (Delgado et al. 2004). VIP downregulates the production of several proinflammatory cytokines, including TNFα, IL-1, IL-6, IL-12, and IFNγ, while stimulating production of anti-inflammatory cytokines IL-10, IL-1R antagonist, and TGFβ (Delgado et al. 1999a; Martinez et al. 1998; Delgado et al. 1999b). Investigation of the effect of VIP in a murine endotoxin challenge model showed that after treatment with VIP, levels of TNFα and IL-6 in serum and peritoneal fluid were reduced by almost 50 % (Delgado et al. 1999c). Regarding the eye, VIP treatment converted the susceptible phenotype (corneal perforation) to resistant (no perforation) in a mouse model of *P. aeruginosa*-induced infection via downregulation of proinflammatory mediators, upregulation of anti-inflammatory molecules, and modulation of host inflammatory cell activation (Szliter et al. 2007).Thus VIP, a 28-amino acid peptide, delivered by several types of neurons to immune organs and lymphoid tissues in the heart, gastrointestinal tract, lungs, kidney, cornea, and skin is anti-inflammatory in bacterial keratitis (Henning and Sawmiller 2001). In fact, evidence indicated a differential response to VIP between infected BALB/c (more) and C57BL/6 (less) mice due to disparate VIPR1 expression by macrophages (which can be induced in a dose-dependent manner by VIP itself) (Szliter et al. 2007). Macrophages are known to play a key role in regulating/balancing pro- and anti-inflammatory activity in the resistant (BALB/c) and susceptible C57BL/6 murine models; therefore, evidence that VIP influences the functional behavior of these cells suggests a key role for this neuropeptide in regulating inflammation and restoration of immune privilege.

4.4 Substance P (SP)

SP is an inflammatory neuropeptide that is found in the naïve quiescent eye (Catalani et al. 2004). SP is involved in the pathophysiology of inflammatory

diseases and has been implicated because of aberrant levels of SP and SP-containing nerve fibers, as well as its receptor, NK1R, in diseased tissues (Marriott and Bost 2001). SP has been shown to elicit cytokine secretion from mouse T cells (Levite 1998). In addition, it was demonstrated that human bronchial epithelial cells produce IL-6, IL-8, and TNFα after SP treatment. SP-induced cytokine production and secretion by leukocytes, including T cells, macrophages, and dendritic cells, lead to the release of a number of inflammatory mediators such as additional cytokines, oxygen radicals, arachidonic acid derivatives, and histamine, all of which further amplify the inflammatory response (Holzer and Holzer-Petsche 1997). VIP directly interferes with the Substance P signaling pathway (Delgado et al. 2004).

4.5 Complement System

Activation of the complement cascade contributes to both inflammation and tissue destruction. It is therefore not a surprise that there are complement regulatory factors in the ocular fluids. Complement regulatory factors regulate complement and its activation. The system is a major component of innate immunity, and therefore, during an inflammatory reaction, the eye is potentially threatened by homologous complement attack, and unregulated complement activation would lead to tissue damage and vision loss (Sohn et al. 2007). The complement system is continuously activated at low levels in the normal eye, but the intraocular complement regulatory proteins limit the effect. In the human, the complement regulatory proteins, membrane cofactor protein (MC, CD46), decay-accelerating factor (DAF, CD55), and membrane inhibitor of reactive lysis (MIRL, CD59) all prevent tissue bystander damage, after complement activation. All of these proteins have been identified in rodents (Sohn et al. 2007; Goslings et al. 1998). In addition, a unique rodent transmembrane protein known as Crry (complement-receptor 1-related gene/protein y) and the homologue for human CD46 (Masli et al. 2006; Streilein 2003) and CD55 act early in the complement cascade to disable the central amplification enzymes C3 and C5 convertases. CD59 functions later in the cascade to prevent membrane attack complex formation by inhibiting the incorporation of C9 (Yang et al. 2009; Kim and Song 2006). Thus spontaneous complement activation is tightly regulated, so there is elimination of potential pathogens without the induction of intraocular inflammation. Alternatively, complement activation proteins like iC3b that are released during the early phase of antigen and antigen-presenting cell contact are essential for the induction of systemic tolerance to antigen injected in the anterior chamber. Thus, on one hand, complement provides innate immunity against pathogens, but, on the other, complement instructs the adaptive immune response to develop tolerance, thus providing host defense without inflammation.

5 Immune Regulatory Molecules Expressed by Ocular Stromal and Innate Immune Cells

5.1 CD200/CD200R

The CD200-triggered CD200R signaling system regulates the ability of local myeloid cells to become activated (Copland et al. 2007; Broderick et al. 2002). CD200R belongs to the super immunoglobulin family. Similar to the intestine and the lung, the eye controls the myeloid cells from activation by CD200 that is produced by local stromal cells. CD200 is a non-signaling molecule that has been described on neurons and belongs to the immunoglobulin superfamily of glycoproteins. Interaction of CD200 with its ligand, CD200R, induces tyrosine phosphorylation (Chitnis 2007). When CD200 binds to its receptor (CD200R) on myeloid cells, it suppresses the ability of the myeloid cells to become activated (Copland et al. 2007; Robertson et al. 2002; Dick et al. 2003). Originally, CD200R was thought to be selectively expressed by myeloid cells; more recent data shows that this regulatory molecule is expressed on select lymphocytes and neutrophils (Minas and Liversidge 2006; Chitnis et al. 2007). CD200 has been found on stromal cells in the eye and is upregulated during inflammatory conditions (Copland et al. 2007; Broderick et al. 2002; Dick et al. 2003). CD200R is expressed by ocular myeloid cells including microglia and F4/80-positive macrophages (Dick et al. 2003).

5.2 Toll-Like Receptors

The regulation of complement receptors also contributes to the regulation of the TLRs. Recognition of invading organisms by the innate immune system is achieved by pattern recognition receptors (PRR) that are specific for unique pathogen-associated molecular patterns (PAMPs) (Akira et al. 2006). TLRs are PRR that are specific for PAMPs. TLRs are broadly distributed on cells of the immune system and, once triggered by pathogen detection, induce innate immunity and help to instruct/strengthen adaptive immunity (Medzhitov et al. 1997). There are 11 identified TLRs in the human (Liew et al. 2005). While TLR activation is required for initiating innate immune response, members of the receptor family are also involved in pathogenesis of autoimmune, chronic inflammatory and infectious diseases (Cook et al. 2004). Thus, the TLR system is tightly regulated.

In addition to endogenous regulatory factors to control TLR-induced inflammation (Liew et al. 2005), there are factors within the eye that limit their constitutive and induced expression. TLRs are differentially expressed by cells within the eye but are often readily induced in culture environments (Fang et al. 2010; Jiang et al. 2009). Few studies have actually studied the effect of the ocular environment on the TLR in the eye (Taylor et al. 2000). It is known that complement metabolites like

C3a and C5a enhance TLR-induced inflammation (Zhang et al. 2007). Thus, when the C-derived anaphylatoxins are inhibited, TLR-induced inflammation is lessened. This is another regulatory mechanism that allows for protection of the eye.

Mouse eye infection models also have been used to study the role of TLRs in disease. This family of receptors, composed of transmembrane molecules, links the extracellular compartment where contact and recognition of microbial pathogens occur and the intracellular compartment where signaling cascades leading to cellular responses are initiated. Gene array data showed that the expression of TLRs and related molecules including CD14, soluble IL-1rα, TLR-6, and IL-18R-accessory-protein was significantly elevated in susceptible (C57BL/6) vs. resistant (BALB/c) mice following challenge with *P. aeruginosa* (Huang and Hazlett 2003). TLR4 was found to be required for the resistance response of BALB/c mice to *P. aeruginosa* challenge (Huang et al. 2006a), in contrast with a sterile keratitis model, where the absence of TLR4 improved outcome (Khatri et al. 2002).

Negative regulators of TLR also are of importance, and recent evidence showed that one of them, single Ig IL-1R-related molecule (SIGIRR), is differentially expressed in BALB/c (resistant) vs. C57BL/6 (susceptible) mice (Huang et al. 2006b). This Toll receptor is critical in resistance to *P. aeruginosa* infection in BALB/c mice, functioning to downregulate type 1 immunity and negatively regulating sustained IL-1 and TLR4 signaling. ST2, a member of the TLR/IL-1R superfamily, was also found to be required for resistance, essentially by upregulating type 2 immunity and in particular IL-10 production (Huang et al. 2007). Most recently, evidence has been provided (Sun et al. 2010) that in mice *P. aeruginosa* activates TLR4/5 on resident corneal macrophages, which signals through Toll/IL1-R intracellular domain (TIR) containing adaptor inducing IFN-β (TRIF) and TIR containing adaptor protein/MyD88 pathways, leading to NFκB translocation and transcription of chemokines, PMN recruitment to the corneal stroma, bacterial killing, and tissue damage. IL-1 is also produced, confirming past reports (Rudner et al. 2000), activating an IL-1R1/MyD88 positive feedback loop in macrophages and IL-1R on other resident cells of the cornea. It has also been shown that bacterial flagellin suppresses the inflammatory response and enhances bacterial clearance in a murine model of *P. aeruginosa* keratitis (Kumar et al. 2008). This is facilitated via flagellin-induced corneal antimicrobial peptide production and wound repair involving an NF-κB-independent and EGFR-dependent pathway (Gao et al. 2010).

5.3 Apoptosis (Green et al. 2009)

Apoptosis is the process of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes and death (Green 2005). Unlike necrosis, apoptosis produces cell fragments called apoptotic bodies that phagocytic cells engulf and rapidly remove, preventing damage to the surrounding cells and tissues.

Activated T cells express ligands for receptors on the ocular pigmented cells that line the borders of the eye that can induce apoptosis of invading cells. Two such apoptotic molecules expressed by the activated T cells are programmed death (PD)-1 and FAS that bind programmed death-ligand 1 (PD-L1) and FasL, respectively, on the pigmented cells that line the eye (Usui et al. 2008; Sugita et al. 2009a) PD-L1 is expressed by corneal endothelial cells (Sugita et al. 2009b) as well as retinal pigmented epithelial cells (Usui et al. 2008; Sugita et al. 2009a) and can induce apoptosis as well as regulate chemotaxis in cells expressing PD1(Hori et al. 2006; El Annan et al. 2010).

As indicated above, apoptotic cells are quickly taken up by local phagocytes. The dying cells release molecules that downregulate the inflammatory and immune response. Apoptotic cells also produce IL-10, an immunosuppressive cytokine that can by itself induce behavioral changes, either directly or indirectly, in cells toward immunosuppression and anergy (Griffith et al. 1996; Müller et al. 2002; Ferguson et al. 2011).

Delayed apoptosis in bacterial keratitis, as evidenced by TUNEL staining, also contributes to corneal perforation in C57BL/6 mice. Consistent with this finding, Bcl-2, an anti-apoptotic gene, was significantly upregulated in C57BL/6 mouse cornea at 18 h p.i., suggesting that the delayed onset of apoptosis in C57BL/6 mouse cornea may be, in part, due to upregulation and signaling of this gene (Zhou et al. 2008). These data also are consistent with previous studies showing that overexpression of Bcl-2 reduces lymphocyte apoptosis in *P. aeruginosa*-induced pneumonia (Coopersmith et al. 2003).

In this regard, the neuropeptide SP, mentioned above, is a potent anti-apoptotic regulator and can exacerbate inflammation. SP has been shown to stimulate phosphorylation of the anti-apoptotic molecule Akt in colonic mucosa (Koon et al. 2007) both in vivo and in vitro, preventing apoptosis in humans with colitis. SP delays spontaneous apoptosis of PMN in a dose-dependent manner by its interaction with the NK1R in vitro, and this effect could be inhibited by application of the NK1R antagonist GR82334 (Bockmann et al. 2001). C57BL/6 mice treated with another NK1R antagonist, Spantide I, showed significantly improved disease outcome and an earlier onset of PMN apoptosis, similar to the pattern observed in naturally resistant BALB/c mice. These data suggest that the protective mechanism of Spantide I treatment in C57BL/6 mice involves less PMN-induced bystander tissue damage and regulation by macrophages (Hazlett et al. 2007).

Macrophages also may provide distinct activation signals for Th1/Th2 differentiation. In this regard, others have reported that *Leishmania major*-infected macrophages enhanced the proliferation and IL-4 secretion of Th2 T cells but inhibited the response of Th1 T cells {Chakkalath, 1994 #8665}. Similarly, we detected that macrophages from C57BL/6 mice expressed significantly more IL-12, while BALB/c cells expressed more IL-10 after LPS stimulation. IL-10 appears protective in the BALB/c-infected cornea, as after subconjunctival injection of clodronate-containing liposomes to deplete these cells, higher levels of IFN-γ and lower levels of IL-10 were detected and resistant mice were converted to the susceptible phenotype (McClellan et al. 2003). It was also reported that

VIP-treated C57BL/6 mice showed improved disease outcome and increased IL-10 expression after *P. aeruginosa* corneal infection (Szliter et al. 2007). Furthermore, the data suggest that SP, which acts in an anti-apoptotic manner toward activated C57BL/6 mouse macrophages, may also enhance an IL-12-driven, Th1-type immune response and thus further contribute to the susceptibility of this mouse strain to *P. aeruginosa* infection (Zhou et al. 2008).

5.4 Defensins

Mammalian antimicrobial peptides, including the defensins, represent an ancient arm of the innate immune system whose role is to directly neutralize invading microbes (Hazlett and Wu 2011). In this regard, studies by Wu et al. (Wu et al. 2009) provided evidence that murine beta-defensin 2 (mBD2) silencing significantly enhanced the mRNA expression levels of IFN-γ, MyD88, and NF-κB at various times (both early and late) after infection, which may be crucial in mBD2-dependent ocular defense against *P. aeruginosa* infection. For other proinflammatory cytokines/molecules (e.g., MIP-2, IL-1β, TNF-α, IL-6, and iNOS) and TLRs (e.g., TLR4 and TLR2), silencing mBD2 led to a shift in mRNA expression: a downregulation at an earlier period, followed by an upregulation at 5 days p.i. This shift is specific to mBD2 silencing, as evidence was provided to confirm both the specificity and effectiveness of silencing using RT-PCR of infected cornea. Nevertheless, other studies have demonstrated that HBD2 and HBD3, the inducible human homologues of mBD3 and mBD4 (Bals 2000; Chong et al. 2008), play an important role in the ocular immune defense system. They do so by regulating a variety of immune events, including bacterial killing, cytokine release, mast cell histamine release, dendritic cell activation, immune cell chemotaxis, as well as epithelial cell migration and wound repair (Tomita and Nagase 2001; McDermott 2004; McDermott 2009; Schroder and Harder 1999; Feng et al. 2005; Garcia et al. 2001; Niyonsaba et al. 2007), suggesting that their homologues could be significant in murine ocular immunity. Moreover, in vitro studies have demonstrated synergistic activities of HBDs against *S. aureus* and *E. coli* (Chen et al. 2005), indicating a significant potential for mBDs to act together to protect the ocular surface from invading pathogens. The expression and function of mBD3 and mBD4 in susceptible B6 vs. resistant BALB/c mice and whether either defensin interacted with mBD2 also were tested. Data (Wu et al. 2009) provided evidence that mBD3 and mBD4 are inducibly and disparately expressed in BALB/c vs. B6 corneal epithelium and stroma after *P. aeruginosa* infection. Of the two, only mBD3 was required for host resistance and interacted with mBD2 to protect against bacterial infection. In vivo studies identified the cell sources of mBD2 and mBD3 in the corneal stroma after infection and demonstrated that despite their individual effects on disease outcome (e.g., iNOS expression), these two defensins act together to promote host resistance against corneal

infection. This is achieved through modulation of bacterial load and regulation of both PMN infiltration and production of proinflammatory cytokines and TLR signaling molecules.

5.5 Immune Privilege and Infection in the Cornea

Visual acuity depends on the clarity of the cornea and other tissues that compose the visual axis. Among the factors that contribute to corneal clarity are an optimal geometric arrangement of collagen fibrils in the corneal stroma and an absence of blood and lymphatic vessels within the normal cornea. However, the angiogenesis and edema that typically accompany inflammatory responses can transiently disrupt corneal clarity through neovascularization and disorganization of collagen fibrils. Additionally, inflammatory mediators can effect changes in the keratocytes that produce the extracellular matrix of the corneal stroma, leading to scarring and permanent vision loss. To maintain clarity, the cornea has adopted a variety of active and passive mechanisms to inhibit inflammation.

Corneal grafts that are placed on an avascular corneal bed enjoy the same 5-year rate of acceptance as other transplanted tissue without the aid of tissue typing and systemic immunosuppression (Hori and Niederkorn 2007). Several inhibitory factors produced in the cornea and adjacent anterior chamber appear to contribute to the immune privilege of the cornea. These include TGF-β, αMSH, and Fas ligand (CD95L) expression on corneal cells (Streilein et al. 2000). Additionally, the lack of blood and lymphatic vessels in the normal cornea limits communication with the immune system (Cursiefen 2007). Together, these features of the cornea inhibit immunoinflammatory reactions by limiting the afferent delivery of antigens from the cornea to adjacent lymphoid organs and by neutralizing T cell effector mechanisms within the cornea.

Given the impressive array of anti-inflammatory mechanisms employed by the cornea and its direct interface with the environment, one might predict that this tissue would frequently fall prey to a variety of environmental pathogens. This is not the case. Moreover, when corneal infections do occur, immunopathology rather than immune deficiency can be the primary cause of the tissue destruction associated with the infection. Here we discuss two such examples involving infection of the cornea with *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) and the ensuing immunopathologic processes that are referred to as Pseudomonas and Staphylococcus keratitis.

5.6 Pseudomonas Keratitis

Pseudomonas aeruginosa is primarily a nosocomial pathogen. According to the CDC, the overall incidence of *P. aeruginosa* infections in U.S. hospitals averages

about 0.4% (4 per 1,000 discharges), and the bacterium is the fourth most commonly isolated nosocomial pathogen accounting for 10.1% of all hospital-acquired infections. *P. aeruginosa* also is an important Gram-negative pathogen associated with bacterial keratitis. It is often referred to as an opportunistic organism, as it is capable of inducing keratitis not only in extended-wear contact lens users but generally in more tropical climates and in patients that are either debilitated or hospitalized. Most complications associated with bacterial keratitis are structural alterations of the cornea, but other sight-threatening problems include development of secondary glaucoma and cataract. These consequences are largely caused by the host's inflammatory response and the abrogation of immune privilege (Hazlett 2007).

P. aeruginosa, like most other microorganisms, requires surface injury to permit corneal invasion. Pseudomonal and other Gram-negative bacterial infections often present as a rapidly progressing infection with a marked mucopurulent exudate. A ring infiltrate may appear in the surrounding paracentral cornea, and hypopyon (a dense inflammatory coagulum in the anterior chamber) is usually present; in addition, descemetocele formation or corneal perforation may occur. Animal models of bacterial keratitis continue to be valuable in the study of this disease and are produced by topical bacterial application after abrading the epithelium by intrastromal inoculation or by placing a contaminated suture or contact lens on the cornea (Hazlett 2002; Szliter et al. 2002). These approaches and models have led to an increased understanding of the mechanisms of corneal inflammation and innate immunity that are operative in the abrogation of immune privilege after infection.

Bacterial eradication by neutrophils (PMN) involves phagocytosis, lysosomal degranulation, and bacterial killing within the acidic lysosomal compartment of the cell. PMN also produce toxic oxygen metabolites; triggering of the respiratory burst results in biosynthesis of superoxide anions and other oxidizing agents such as hydrogen peroxide. Phagocytic secretion and lysis result in release of extracellular lysosomal enzymes, including, but not limited to, elastase, collagenase, and myeloperoxidase (MPO). These enzymes and the oxygen-derived free radicals cause stromal destruction by breaking down collagen, digesting glycosaminoglycans, and disrupting stromal keratocytes. Nitric oxide (NO) also mediates vasodilation and can be important in bacterial killing as well as in bystander tissue damage. These and other substances released from activated PMN and other inflammatory cells (e.g., macrophages) contribute to stromal necrosis and corneal edema during infection. Bacterial endotoxin and exotoxins also stimulate macrophages to release biologically active substances including IL-6 and TNFα, cytokines that synergize to elicit inflammation (Wilhelmus 1996). Maintenance of leukocyte recruitment, a common feature during inflammatory processes (Strieter et al. 1996), requires intercellular communication between infiltrating leukocytic cells, the epithelium, neuropeptides such as SP and VIP, vascular endothelium, and resident stromal cells. Such events are mediated by generation of early response cytokines (e.g., IL-1), the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), and the production of chemotactic cytokines and chemokines.

5.7 Neutrophils, Cytokines, and Chemokines

If leukocytes such as PMN persist within the cornea, destructive pathology occurs, including stromal scarring and perforation, the latter often requiring corneal transplantation (Hazlett 2004). PMN infiltration into inflamed tissue is controlled largely by local production of inflammatory mediators. In the mouse, two members of the CXC family of chemokines, MIP-2 (functional homologue of human IL-8) and KC, are potent chemoattractants and activators of PMN (Driscoll 1994). In corneal infections, MIP-2 has been shown to be the major chemokine that attracts PMN into the *P. aeruginosa*-infected cornea, and their persistence in the cornea of susceptible (cornea perforates) C57BL/6 vs. resistant BALB/c (no corneal perforation) mice was found to correlate with higher MIP-2 chemokine levels (both mRNA and protein) (Kernacki et al. 2000). Furthermore, after infection, matrix metalloproteinase 9 (MMP-9) was shown to upregulate chemotactic cytokines/chemokines (IL-1β and MIP-2), contribute to degradation of collagen type IV, and, overall, enhance *P. aeruginosa* keratitis (McClellan et al. 2006). In contrast, neutralization of IL-1β in infected C57BL/6 mice reduced disease severity, evidenced by reduction of PMN in the cornea (measured by MPO assay), decreased bacterial load (plate count), and decreased levels of MIP-2 at both the mRNA and protein levels (Rudner et al. 2000). The use of caspase 1 (an enzyme that processes IL-1β to generate the mature cytokine) inhibitor treatment in C57BL/6 mice confirmed these data, even when inhibitor treatment was initiated 18 hours after induction of experimental disease. In addition, improvement was augmented when the caspase 1 inhibitor was given after infection together with the antibiotic ciprofloxacin (Thakur et al. 2004).

5.8 Staphylococcus Keratitis

Staphylococcus aureus (*S. aureus*) is a Gram-positive, cluster-forming coccus that is nonmotile and nonsporeforming. It is a facultative anaerobe that ferments mannitol, and it is both catalase and coagulase positive. The bacterium is part of the normal flora of humans found on nasal passages, skin, and mucous membranes, but it is also a pathogen of humans, capable of causing a wide range of suppurative infections, as well as food poisoning and toxic shock syndrome. *S. aureus* is the leading cause of bacterial keratitis in adults, including those who have sustained penetrating corneal injuries or are compromised by immunodeficiencies (Asbell and Stenson 1982). Tissue damage during bacterial keratitis results from the action of bacterial products on ocular tissues as well as the host inflammatory response to the infection (Callegan et al. 1994; Hazlett et al. 1992; Sun et al. 2006). When *S. aureus* penetrates the corneal epithelium and the corneal stroma, there is rapid bacterial replication, production of toxins, including hemolytic α-toxin or as below

alpha, and severe tissue damage, leading to corneal opacity. *S. aureus* infection also stimulates extensive PMN infiltration to the corneal stroma, with subsequent degranulation and release of cytotoxic mediators further contributing to the pathogenesis of this disease (Callegan et al. 1994). *Staphylococcus* keratitis can result in irreversible corneal scarring, resulting in a loss of visual acuity. Multidrug-resistant strains of the bacterium further complicate therapy of these infections (Moreira and Daum 1995), particularly with the increased incidence of infections by methicillin-resistant strains (Kreiswirth et al. 1983; Balaban and Novick 1995).

5.9 Toxins

Past studies have examined the roles of specific staphylococcal proteins (alpha-toxin and protein A) in corneal virulence in an experimental rabbit model of keratitis (Callegan et al. 1994). Alpha-toxin-producing (Hla+) strains caused significantly greater ocular inflammation and corneal damage than alpha-toxin-deficient Hla strains. Hla strains produced significantly less inflammation of the conjunctiva and iris and almost no corneal epithelial erosion or stromal ulceration.

The pore-forming alpha-toxin damages cell membranes, an activity suggested to contribute to tissue damage in several models of infection (Callegan et al. 1994; Bramley et al. 1989; Menzies and Kernodle 1994). Beta-toxin, a sphingomyelinase, is a possible virulence factor responsible for tissue necrosis during experimental murine mastitis (Bramley et al. 1989). The lytic action of beta-toxin is known to be limited by the sphingomyelin content of cell membranes (Bernheimer 1988; Bernheimer et al. 1974). Corneal and scleral epithelial cell membranes have a high sphingomyelin content (Broekhuyse 1975). Therefore, both tissues could be targets for beta-toxin during ocular infection.

Another study used genetic rescue experiments and confirmed the role of alpha-toxin as a major virulence factor during *S. aureus* keratitis and implicated beta-toxin, a mediator of edema, as a lesser contributor to ocular damage. The study also showed that application of purified alpha-toxin produced corneal epithelial erosions and iritis, while application of beta-toxin caused scleral inflammation (O'Callaghan et al. 1997).

5.10 TLR

S. aureus is readily killed by components in tears, especially phospholipase A2 (Moreau et al. 2001), but bacterial products can stimulate a local inflammatory response (Johnson et al. 2005; Schultz et al. 1997). In a murine model of *S. aureus* keratitis, topical application of 1×10^8 bacteria to scarred corneas caused keratitis in A/J and BALB/c, though not C57BL/6, mouse strains as measured by slit lamp examination (Girgis et al. 2003). Other studies using the latter mouse strain

demonstrated that bacterial products activate the TLR family of pathogen recognition molecules on corneal epithelial cells to produce CXC chemokines, which then facilitate PMN recruitment into the corneal stroma, leading to corneal haze (Khatri et al. 2002). Three members of the TLR family, TLR2, which binds lipoproteins; TLR4, which binds bacterial lipopolysaccharide (LPS); and TLR9, which binds unmethylated CpG-rich DNA in the mouse corneal epithelium with signaling through the common adaptor molecule myeloid differentiation factor 88 (MyD88), were tested (Sun et al. 2006). It was shown that clinical and laboratory strains of *S. aureus* induced PMN recruitment to the corneal stroma and increased corneal haze and thickness; that corneal inflammation and CXC chemokine production are dependent on TLR2 and MyD88, but not on TLR4 or TLR9; and that *S. aureus*-induced PMN activation is dependent on TLR2. Consistent with the previous study (Girgis et al. 2003), these authors saw no changes by slit lamp examination in challenged mice; however, when the early subclinical responses were tested using in vivo confocal microscopy, they found consistent increases in stromal thickness and haze in response to *S. aureus* that were dependent on functional TLR2 and MyD88.

5.11 Protein A

The ability of *S. aureus* to establish infections in a wide range of body sites probably depends on synthesis of a large number of extracellular and cell-bound virulence factors. Among these factors, protein A (SpA), a 42-kDa molecule existing in a secreted and membrane-associated form, interacts with a variety of human and animal immunoglobulins (Silverman and Goodyear 2006). The role of SpA as a virulence factor of *S. aureus* in various staphylococcal diseases including that in keratitis has been documented (Callegan et al. 1994; Foster 2005). SpA also induces an inflammatory response in human corneal epithelial cells (HCECs) characterized by secretion of proinflammatory cytokines and chemokines by a mechanism distinct from that of TLRs such as TLR2 (Kumar et al. 2006; Kumar et al. 2004). This response is characterized by activation of multiple signaling pathways (NF-κB, p38, and ERK) and production of proinflammatory cytokines/chemokines such as TNF-α and IL-8. In contrast, the possible contribution of SpA to *S. aureus* virulence has also been investigated in a rabbit keratitis model, and this study has shown that SpA does not contribute significantly to the severity of the disease (Callegan et al. 1994).

5.12 Lipoprotein

The response of HUCL, a telomerase-immortalized human corneal epithelial cell (HCEC) line, to lipoproteins (LP) isolated from *S. aureus* also has been tested

(Kumar et al. 2007). *S. aureus* LP (saLP) prepared by Triton X-114 extraction stimulated the activation of NF-κB, JNK, and p38 signaling pathways in HUCL cells. The extracts failed to stimulate NF-κB activation in HUCL cells after lipoprotein lipase treatment and in cell lines expressing TLR4 or TLR9, but not TLR2, indicating the lipoprotein nature of the extracts. saLP induced the upregulation of a variety of inflammatory cytokines and chemokines (IL-6, IL-8, ICAM-1), antimicrobial molecules (hBD2, LL-37, and iNOS), and homeostasis genes (Mn-SOD) at both the mRNA and protein level. A similar inflammatory response to saLP was also observed in primary cultured HCECs. Moreover, TLR2-neutralizing antibody blocked saLP-induced secretion of IL-6, IL-8, and hBD2 in HUCL cells, suggesting that saLP activates TLR2. These studies compliment other work in the mouse suggesting that TLR2 is important in disease pathogenesis (Sun et al. 2006).

Thus, immunoregulatory mechanisms of the eye allow for defense against infections in the absence of inflammation. These mechanisms are multiple and overlapping and include physical barriers, apoptosis, and modulating approaches to controlling invading immune cells. In spite of the elaborate regulatory pathways, infections do occur in the eye but in most cases are quickly controlled, and the immune homeostasis in the eye is restored. Most therapy for infections is based on antibacterial approaches. It is unlikely that therapy to induce strong immune responses to organisms in the eye would be useful because of the risk of inflammation damaging tissue and the ability to see.

6 Current Therapeutic Approaches

6.1 P. Aeruginosa

P. aeruginosa is often resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and fluoroquinolones, resistant forms have developed. The combination of gentamicin and carbenicillin is frequently used to treat severe *Pseudomonas* infections (Table 2). Several types of vaccines are being tested, but none is currently available for general use.

Beginning with the use of penicillin in the 1940s, drug resistance has developed in the staphylococci within a very short time after introduction of an antibiotic into clinical use. Some strains are now resistant to most conventional antibiotics, and there is concern that new antibiotics have not been forthcoming. New strategies in the pharmaceutical industry to find antimicrobial drugs involve identifying potential molecular targets in cells (such as the active sites of enzymes involved in cell division) then developing inhibitors of the specific target molecule. Hopefully, this approach will turn up new antimicrobial agents for the battle against staph infections. Indeed, since 2003, alternatives to vancomycin have been approved for treatment of MRSA.

No vaccine is generally available that stimulates active immunity against staphylococcal infections in humans. A vaccine based on fibronectin binding protein

Table 2 Antibacterial therapy of *P. aeruginosa* and *S. aureus*

Bacteria	Antibiotic	Topical concentration	Subconjunctival dose
Staphylococcus species	Cefazolin	50 mg/ml	100 mg in 0.5 ml
e.g., *S. aureus*	Vancomycin[a]	15–50 mg/ml	25 mg in 0.5 ml
	Tobramycin/gentamicin	9–14 mg/ml	20 mg in 0.5 ml
Pseudomonas species	Ceftazidime	50 mg/ml	100 mg in 0.5 ml
e.g., *P. aeruginosa*	Fluoroquinolones	3 mg/ml	100 mg in 0.5 ml

[a]For resistant Staphylococcus species and penicillin allergy

induces protective immunity against mastitis in cattle and might also be used as a vaccine in humans. However, vaccine therapies represent a new and innovative approach in broadening the available clinical tools against the global health problem of community and healthcare-associated *S. aureus* bacterial infections. Clinical treatment is summarized for both of these pathogens in Table 2 which has been modified from its original source (Zhou et al. 2008).

In summary, both *P. aeruginosa* and *S. aureus* share common features in that in keratitis, they elicit a response through TLR receptor engagement, attract PMN into the cornea, and activate transcription factors such as NFκB which in turn promote a cytokine/chemokine response in the cornea that if left uncontrolled is devastating and sight threatening. Models that allow testing of the pathobiological processes involved during infection may reveal targets for therapy for both of these eye diseases and remain critical for rational development of novel therapeutics to treat blinding eye diseases.

7 Ocular (Adaptive) Immune Privilege

An important concept for understanding the adaptive immune response is that antigen alone will not activate lymphocytes. Lymphocytes that bind to the antigen-presenting cells with their antigen-specific receptor (T cell receptor, TCR) need a second signal to proliferate and differentiate into effector cells. The T cell that does not receive the second signal may become anergic or unable to respond. Examples of second signals include adjuvants, bacterial products, and cytokines. Bacterial products and stress proteins bind the TLRs to provide second signals during an effective immune response. Trauma induces heat shock proteins that can be immunogenic, act as adjuvants, and bind to TLRs, providing necessary signals for lymphocyte activation.

For a primary response, activation of lymphoid cells takes place in the secondary lymphoid organs and never in the tissue itself. Thus the antigen is transported to the lymphoid organ where it is presented to the lymphocytes, and T and B cells begin to proliferate and differentiate into effector cells that then migrate back to the site of antigen. This process takes at least 5–7 days to complete. In case of the anterior chamber, the secondary lymphoid organ is the spleen, but in respect to the conjunctiva, the draining lymph nodes are involved in the activation of the immune

response, and therefore, immune responses begin with antigen presentation in the T cell areas of the lymph node.

8 Mechanisms That Stop Activated Immune Cells from Invading and Damaging the Eye

8.1 Physical Barriers (Fig. 1)

The eye is structurally configured so cells cannot migrate from the blood into the retina. This is accomplished by tight junctions between the endothelial cells and is called the blood–ocular barrier. Local inflammatory processes can, however, induce leakiness in the vascular and epithelial barrier to allow cells from the blood access to the eye. In such cases, other mechanisms contribute to preventing inflammation.

8.2 Modification of Behavior

There are a variety of pathways that mediate cellular behavior changes that are triggered by surface molecules expressed by both stromal and immune cells. The pigmented cells lining the eye also have the capacity to induce a behavioral change in the activated cell. Activated T cells express CTLA4 that is capable of binding to B7 expressed by the pigmented stromal cells (Sugita 2009). This ligation induces T effector cells to become T regulatory (Treg) cells (Usui et al. 2008; Sugita et al. 2009a; Sugita et al. 2009b; Hori et al. 2006; El Annan et al. 2010; Sugita et al. 2008).

8.3 Immunosuppressive Environment of the Eye

The immunosuppressive molecules in the fluids of the eye also downregulate the adaptive immune system. Since indigenous antigen-presenting cells are exposed to TGFβ 2, they do not express the necessary coreceptors for activating an immune response but rather produce immunosuppressive factors. This change in function allows the eye-derived antigen-presenting cells to process and present antigens to naïve T cells in lymphoid organs in a way that favors T cell differentiation into antigen-specific Treg cells (Wilbanks and Streilein 1992). In addition the multiple soluble immunosuppressive factors have direct effects on stray activated cells that wander into the ocular environment.

9 Mechanism That Induces Local and Peripheral Tolerance to Ocular Antigens

Anterior chamber-associated immune deviation (ACAID) was first defined by studies of Kaplan and Streilein in the early 1970s (Kaplan et al. 1975). They observed that antigens inoculated into the anterior chamber of the eye induced a peripheral tolerance to that antigen. The phenomenon was called anterior chamber-associated immune deviation because it quickly became apparent that the eye was not without any immune response but lacked only those that were inflammatory (Wilbanks and Streilein 1990a; Wilbanks and Streilein 1990b). Inflammation would cause a serious disruption to the visual axis. Much has been learned in the 40 years since the original report of the ACAID model for tolerance.

It is known that the antigens inoculated into the anterior chamber are transported to the marginal zone of the spleen by the indigenous F4/80+ macrophages (Streilein and Wilbanks 1990; Wilbanks and Streilein 1991). The F4/80 APC secrete chemokines that recruit the invariant NKT cells to the site where they interact with marginal zone B cells and T cells (Faunce et al. 2001) to induce the differentiation of both afferent CD4+ Treg cells and efferent CD8+ Treg cells (Wilbanks and Streilein 1990b; Sonoda et al. 2001). Within a week post antigen inoculation, the Treg cells are available to suppress the induction and the effector stages of the immune response. More recently, reports by Griffith and colleagues show that antigen injection in to the anterior chamber primes $CD8^+$ regulatory T cells to produce TRAIL and TRAIL-positive $CD8^+$ Treg cells contribute to systemic tolerance to the eye inoculated antigen (Griffith et al. 2011).

The required interactions of the cells that aggregate in the marginal zone have been studied and show that the APC must express F4/80 since F4/80 null mice do not develop ACAID post anterior chamber inoculation of antigen (Lin et al. 2005). Interestingly, this low-dose oral tolerance cannot be induced in F4/80 null mice either (Lin et al. 2005), raising the possibility that the ACAID-inducing mechanisms described in the spleen might be used by the gastrointestinal tract as well. It is known that the F4/80 APC that induces ACAID does not express the major immune coreceptors (CD40, class II) and produces IL-10 and TGFβ, but not IL-12. Importantly, it does not express CCR7, or CCR6, but does express CXCR4. The lack of CCR7 explains why APC do not migrate to the T cell areas of the spleen. The role of CXCR4 in its path to the marginal zone (MZ) has not been studied. Another critical molecule expressed by ACAID APC is the CD1d molecule. CD1d is a class I-like molecule that is known to present lipids to T cells. The T cell receptor (TCR) on the invariant NKT cell that binds the CD1d molecule is required for the cell's development as well as its activation. Thus CD1d-deficient mice do not develop ACAID (Sonoda et al. 1999). It is presumed that during the induction of ACAID tolerance, the invariant T cell receptor (TCR) on the NKT cell recognizes an unknown self-lipid. The marginal zone (MZ) B cells also express CD1d (class I-like molecules that present lipid) (Exley et al. 2011); however, the B cell's role in activating NKT cells is unknown.

A role of B cells in ACAID was first reported by Niederkorn and colleagues. Their publications suggest that the F4/80 APC leaves the eye and transfers its ocularly derived antigen to the splenic B cells that in turn present the antigen to the T cell (D'Orazio and Niederkorn 1998; D'Orazio et al. 2001). The MZ B cell also produces IL-10 and may contribute to the immunosuppressive environment of the cell aggregate in the splenic marginal zone.

iNKT cells are required for the induction of ACAID since mice deficient in the iNKT cells are unable to respond to intracamerally inoculated antigens with an ACAID response (Sonoda et al. 1999). Other studies report that the iNKT cells that induce ACAID are required to express CD4 and LY49 C/I (Watte et al. 2008). While the role of the CD4 marker has not been explored in this model, others have reported that CD1d not only binds the invariant TCR on the iNKT cell but is capable of finding CD4 to activate the iNKT cell (Thedrez et al. 2007). From our laboratory, Watte reported a role for LY49C/I in the production of NKT cell-derived IL-10, an immunosuppressive cytokine necessary for ACAID induction (Sonoda et al. 2001). In the mouse, the majority of the iNKT cell is double negative for the CD4, CD8 markers. Thus the tolerance-inducing population of iNKT cells is described as a minor population of an already rare population of innate cells (Nowak and Stein-Streilein 2007; Yamamura et al. 2007). A deficiency or absence of iNKT cell has been associated with a variety of autoimmune diseases both in the human and the mouse. Thus, the mechanisms involved in ACAID development might contribute to self-tolerance in a variety of organs and systems throughout the body.

10 Ocular Immune Privilege and the Impact of Intraocular Inflammation

Immune privilege is a characteristic of ocular site and tissue. The physiologic mechanism of immune privilege is designed to provide the eye with protection against pathogens while preserving the delicate visual axis from the sight-destroying potential of inflammation. It is often assumed that the presence of intraocular inflammation is incompatible with the existence of immune privilege (Streilein et al. 2002). Streilein and colleagues tested this postulate in several animal models. In one model, the antigen (ovalbumin) that was injected into the anterior chamber of eyes inflamed by mycobacterial adjuvant-induced uveitis or EAU was transiently unable to induce ACAID. It seems that after the initial induction of EAU the immunosuppressive microenvironments reemerged in the inflamed eyes, and at that time, ACAID could be induced. Thus, immune privilege is surprisingly resistant to abolition by intraocular inflammation, and the maintenance of the immune privilege in the face of inflammation depends on the emergence of progressive and partially different immunosuppressive mechanisms.

In contrast, recent reports from our laboratory show that retinal laser burn (RLB) to a very small portion of the retina of one eye abrogated the immune privilege in both eyes for more than 60 days (Qiao et al. 2009). In fact the loss of immune privilege lasted beyond the repair of the wound induced by the retinal burn and the sealing of the blood–brain barrier. Since there is no known direct connection between the eyes, there is a strong possibility of neuronal signals being responsible for upsetting the immune regulation in the eye post retinal laser burn. Interference with potential neuroinflammatory signals post trauma may prevent the long-term loss of immune privilege and subsequent autoimmune disease that might follow retinal trauma.

In summary, we have reviewed the immunoregulatory mechanisms of the eye to allow for defense against infections in the absence of inflammation as well as discussed bacterial organisms that overcome the immunoregulation of the eye to establish infections. In spite of the elaborate regulatory pathways, infections do occur in the eye but in most cases are quickly controlled, and the immune homeostasis in the eye is restored. Most therapy for infections is based on antibacterial approaches. It is unlikely that therapy to induce strong immune responses to organisms in the eye would be useful because of the risk of inflammation damaging tissue and the ability to see.

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Immune Privilege of the Testis: Meaning, Mechanisms, and Manifestations

Mark Peter Hedger

Abstract The mammalian testis belongs among a small number of tissues that can unambiguously be called "immunologically privileged," as demonstrated by the ability to tolerate not only testicular autoantigens but also allo- and xenoantigens experimentally located within the testis environment. The mechanisms underlying this privilege remain poorly understood compared with more intensively studied models of immune privilege, such as the eye and feto-uterine unit, but evidently share key functional elements with these tissues. While physical structures like the blood-testis barrier have been implicated, antigen sequestration, aberrant lymphatics, or impeded immune cell access is not the underlying cause of testicular immune privilege, and it is increasingly evident that privilege involves active immunoregulation and local immunosuppression. More specifically, the unique somatic cells of the testis, the Sertoli cells of the seminiferous epithelium, and the steroidogenic Leydig cells, together with the large resident testicular macrophage population, have been directly implicated in suppressing or regulating immune responses to antigens located within the testicular environment. It is increasingly evident that these immunological control mechanisms also impinge upon, and may even participate in the regulation of, normal testicular function, spermatogenesis, and steroidogenesis. Conversely, failure of immune privilege is a significant cause of disease in the male tract, leading to chronic inflammation, infertility, and pain.

Keywords Autoimmune orchitis • Blood-testis barrier • Hypogonadism • Infertility • Leydig cell • Sertoli cell • Sperm antibodies • Spermatogenesis • Steroidogenesis • Testicular macrophages

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1 Introduction

Maturation of the immune system occurs during fetal and early neonatal life. Arguably the most important aspect of this maturation is acquisition of the ability of the immune system to recognize or, more accurately, to ignore self-antigens. This ignorance involves the deletion or inactivation of antigen-specific T cell and B cell clones that are capable of interacting with antigens that are normally expressed by the host, leading to immunological tolerance of these self-antigens (Nossal 1994; Mueller 2010). Spermatogenesis, which is the production of mature sperm from stem cell precursors in the testis, occurs exclusively within the postpubertal period. Consequently, this is one of the few biological processes whereby large numbers of novel autoantigens appear after the maturation of the immune system, paralleled only by the fetal allograft during pregnancy and the emergence of new antigens that may be associated with tumors and viral infections. Protection of the developing spermatogenic cells from the immune system throughout adult life is a physiological imperative, and the testis has established mechanisms to facilitate this protection. The following review provides a brief outline of the current evidence for testicular immune privilege, the mechanisms underlying this privilege in so far as they are currently understood, and the consequences for testicular function, which may impact upon male fertility and health.

2 Critical Aspects of Testicular Structure and Function

The mammalian testis comprises two functional tissue compartments: the seminiferous tubules, where the spermatogenic stem cells (spermatogonia) develop into mature sperm (spermatozoa), and the interstitial tissue, which contains the steroidogenic Leydig cells, as well as the testicular vasculature and its innervation (Fig. 1). The testicular lymphatics also entirely invest the interstitial tissue, generally comprising discrete lymphatic vessels but forming open lymphatic sinusoids that are contiguous with the interstitial fluid space in some species, most notably the principal laboratory rodents (rats and mice)(Fawcett et al. 1973; Setchell et al. 1990). The seminiferous tubules possess a complex epithelium, comprised of the developing spermatogenic cells and supporting epithelial cells, called Sertoli cells, bounded by a basement membrane and a layer of specialized myoid-like peritubular cells. The tubules are entirely avascular but are closely surrounded by an elaborate and extensive network of arterioles, venules, and capillaries (Suzuki and Nagano 1986; Ergün et al. 1994). Within the seminiferous epithelium, the spermatogonia rest on the basement membrane at the base of the Sertoli cells, slowly undergoing regular mitotic divisions. At precisely defined intervals, the spermatogonia undergo an asymmetric mitotic division, with one daughter cell becoming committed to the process of spermatogenesis. After several further mitotic divisions, the committed spermatogonia enter into meiosis, thereby becoming spermatocytes, and are displaced toward the lumen of the tubule between the adjacent Sertoli cells. Extensive structural reorganization and differentiation of the spermatocytes occurs,

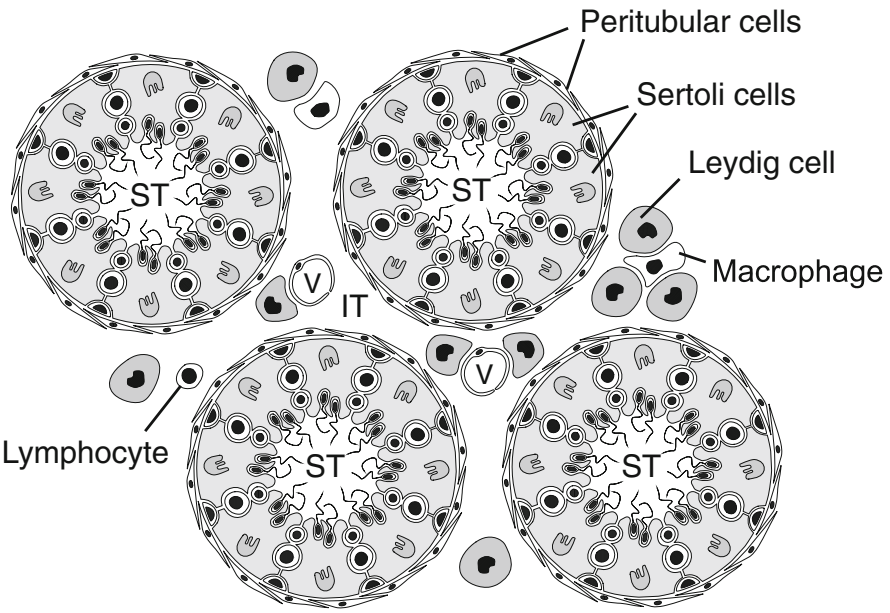

Fig. 1 Diagrammatic cross section of the mature testis, showing the major cellular and structural features relevant to testicular immunoregulation. The testis comprises two major compartments, the seminiferous tubules (ST) and the interstitial tissue (IT). The seminiferous epithelium comprises the epithelial Sertoli cells, with the developing spermatogenic cells lying between these cells and progressing toward the lumen of the tubule as they mature. The tubules are surrounded by a basement membrane and a layer of peritubular cells. Within the interstitial tissue are found numerous Leydig cells, macrophages, and lymphocytes, as well as the testicular blood vessels (V). The testicular lymphatics are also located within the interstitial tissue. In most species, the testicular lymphatics are discrete vessels lined with a continuous endothelium, but in the rat and mouse, they form open sinusoids that are continuous with the interstitial tissue space

producing, after two meiotic divisions, haploid spermatids. The spermatids undergo further elaboration of their cytoplasmic and nuclear contents, including the appearance of several entirely new structures unique to the sperm, such as the acrosome and flagellum, and exchange of most of the nuclear histones for a new set of nucleoproteins, called protamines. As the spermatogenic cells mature through this process, they progress toward the lumen of the tubule, eventually becoming embedded in the apical cytoplasm of the Sertoli cells and are held in place by unique, highly specialized intercellular junctional complexes (Kerr et al. 2006). Within the epithelium, several generations of differentiating spermatogenic cells form multiple cell layers of increasing complexity and development moving toward the tubular lumen. Finally, the Sertoli cells release the spermatids into the lumen, at the same time stripping away and digesting most of the excess cytoplasm of the cell, producing free spermatozoa, which bear little physical resemblance to the spermatogonial stem cells from which they arose. More importantly, in the context of this chapter, the molecular and biochemical composition of these cells is also quite distinct from that of the spermatogonia or any somatic cells of the testis (Chalmel et al. 2007; Aitken and Baker 2008).

Each seminiferous tubule is connected at both ends to a collecting structure within the testicular parenchyma, called the rete testis, which is directly connected to the genitourinary tract via a series of excurrent ducts. These ducts carry the sperm into the adjacent epididymis, where the sperm mature, undergoing further differentiation and modifications that render them capable of movement and fertilization, and are stored prior to ejaculation. At the time of ejaculation, sperm are rapidly propelled from the epididymides via the vasa deferentia into the urethra to join with the secretions of the accessory sex organs, most notably those of the seminal vesicles and prostate. Consequently, the testis and the epididymis are the tissues where large numbers of spermatogenic cells or sperm are present for long periods of time, although sperm also may be found within the lumen of the vas deferens, urethra, and the accessory organs, even outside the time of ejaculation (McClinton et al. 1990; Peirce et al. 2003).

Crucially, spermatogenesis, with the appearance of spermatocytes, spermatids, and spermatozoa in the testis, first occurs at the time of puberty. Puberty is driven by an increase in secretion from the anterior pituitary of the gonadotropins luteinizing hormone (LH), which drives the maturation of the Leydig cells and stimulates testosterone production, and follicle-stimulating hormone (FSH), which stimulates maturation of the Sertoli cell (Plant and Wichel 2006). Both FSH and testosterone regulate the activity of the Sertoli cell, which is essential for the onset of spermatogonial commitment to meiosis and maintenance of spermatogenesis in the adult testis. Prior to puberty, for a period of about 12 years in the human male, the only spermatogenic cells that are present in the testis are the spermatogonial stem cells. Consequently, numerous antigens that are uniquely associated with the developing and mature sperm, as well as many molecules produced by the Sertoli cells during active spermatogenesis, will have escaped the normal mechanisms of tolerance induction, particularly through clonal deletion of lymphocytes, during fetal and early neonatal life and may be seen as foreign by the host immune system. Moreover, at the time of puberty, the Leydig cells undergo extensive transformation from a relatively quiescent precursor into a highly active steroid-producing cell (Nistal et al. 1986). Several enzymes, cellular structures, and other molecules that are unique to mature steroid-secreting cells increase dramatically within the interstitial tissue at this time.

3 Origins and Significance of Testicular and Sperm Autoimmunity

Presumably as a result of this dramatic transformation of the testis at the onset of sexual maturity, many testicular autoantigens appear to be incompletely protected from the immune system. The spermatogenic cells, in particular, are highly immunogenic, as indicated by a relatively high incidence of "spontaneous" fertility-suppressing sperm antibodies in the male tract, affecting approximately one in every 200 men in the developed world (Baker et al. 1983; Lenzi et al. 1997).

In most such cases, leakage of sperm from the male reproductive tract due to congenital malformations, physical trauma, infection-related inflammation, or surgical interventions, such as vasectomy, causes an immunological response, which leads to the formation of sperm antibodies (Linnet 1983; Tung 1987). Genetic predisposition toward autoimmunity also appears to be a significant factor (Baker et al. 1985; Paschke et al. 1994). These antibodies target the sperm for destruction in the male and female reproductive tracts or interfere with their ability to swim toward, bind , and fertilize the egg. Although these antibodies may resolve themselves with time, many affected men experience permanent immunological infertility, which may require assisted reproduction to bypass the antibody-mediated impediments (Clarke et al. 1997). In some animal models, and possibly some human patients, antibody reactions may be followed by autoimmune orchitis and damage to the seminiferous epithelium, potentially resulting in complete sterility (Tung and Alexander 1980; Salomon et al. 1982; Kohno et al. 1983; Roper et al. 1998; Schuppe et al. 2008). Such susceptibility is genetically determined and is most commonly associated with the autoimmune polyglandular syndromes, which are due to genetic disruption of critical tolerance-inducing mechanisms, leading to damage of multiple endocrine organs, targeting steroidogenic cells in particular (Maclaren et al. 2001). Certain infections and inflammation in the male tract, most notably mumps orchitis, are also a potential cause of destructive testicular autoimmunity (Krieger 1984; Philip et al. 2006). The incidence of autoimmune orchitis and resulting infertility in humans appears to be rare, although it is almost certainly considerably underestimated. It is likely, for example, that idiopathic, noninfectious or sterile scrotal, pelvic, or perineal pain, a condition that affects many men particularly in older age, may involve underlying autoimmunity in the reproductive tract, including testicular autoimmunity (Pannek and Haupt 1997; Rivero et al. 2007).

Given the susceptibility of testicular and sperm autoantigens to immunological attack, one may be tempted to ask why responses to these antigens are so relatively infrequent. The answer lies in the fact that the male reproductive tract provides a unique immunological environment for sperm. It is generally accepted that the male reproductive tract constitutes an element of the mucosal immune system and that typical mechanisms responsible for tolerance in other mucosal tissues are involved (Clifton et al. 1992; Czerkinsky et al. 1999; Knee et al. 2005). However, as outlined in the remainder of this chapter, there is considerable evidence that the testis also possesses a number of unique immunological features that single it out for particular interest and have led to its classification as a tissue of specific immunological privilege.

4 The Meaning and Limits of Testicular Immune Privilege

The term “immune privilege” tends to have different meanings for different researchers. The most widely encountered definition is that of a site where lymphatic drainage or immune cell access is restricted, and sequestration of antigens of

the central nervous system behind the blood-brain barrier is usually considered to be the best example of this phenomenon (Carson et al. 2006). However, the original definition of immune privilege more specifically refers to a site where normal rejection responses against foreign tissue grafts, specifically allografts or xenografts, are reduced or prevented (Barker and Billingham 1977; Head and Billingham 1985b). These are functional definitions, promulgated prior to the emergence of modern principles of immunoregulation and tolerance. A more contemporary definition of immune privilege, which would encompass both of these traditional concepts as well as more recent immunobiology, can be stated as "the extended survival of cells expressing antigens that under normal circumstances should provoke an immune response, as well as the mechanisms that contribute to this survival" (Hedger 2007).

Several lines of evidence indicate that the testis is immune privileged by this definition. As outlined in the previous section, the argument from logic is that the developing spermatogenic cells need to be protected from the host immune system. The most direct evidence for immune privilege in the male reproductive tract, however, comes from a number of studies that have shown extended allograft and xenograft survival within the testis. Admittedly, this increased graft survival has only been convincingly demonstrated in laboratory rodents, including rats, mice, and guinea pigs (Ferguson and Scothorne 1977; Bobzien et al. 1983; Head et al. 1983a). Attempts to replicate this in other species, such as sheep and monkeys (Maddocks and Setchell 1988; Setchell et al. 1995), have been less successful, and even in rodents, it is clear that the parameters that underlie successful graft survival into the testis are poorly understood. Various factors, including tissue complexity; vascularization and differences in lymphatic organization; the size, health, and type of graft; the underlying immunogenetics of the donor and host; and even the surgical procedures employed, no doubt influence intratesticular graft success rates. Nonetheless, regardless of the reasons why graft survival has occurred in some studies but not in others, the testis certainly fits the classical criteria of immune privilege as proposed by Billingham and colleagues, as a site where foreign grafts may enjoy extended survival relative to other sites (Barker and Billingham 1977; Head and Billingham 1985b).

There is evidence that testicular tissue and, more specifically, some testicular cells have inherently immunologically privileged characteristics that may make them more amenable to transplantation (Neaves and Billingham 1979; Statter et al. 1988; Barten and Newling 1996). Mouse testis allografts have been reported to survive under the kidney capsule (Bellgrau et al. 1995), and allogeneic transplantation of spermatogenic cells, which were subsequently able to undergo spermatogenic development, have been performed in immunologically intact pigs, goats, bulls, and dogs (Dobrinski 2005; Herrid et al. 2006; Kim et al. 2008). As is the case with grafts into the testis, however, such grafts of testicular tissue have not been universally successful for reasons that are still not understood. More significant is the observation that isolated testicular cell preparations containing Sertoli cells can be successfully transplanted into various tissues across both allogeneic and xenogeneic barriers (Mital et al. 2010). The mechanisms and implications of this unique property of the Sertoli cell will be discussed in more detail later.

5 Mechanisms Underlying Immune Privilege in the Testis

In spite of the absence of most spermatogenic cells at the time of the establishment of central tolerance, mechanisms of tolerance are clearly important for preventing testicular autoimmunity. This is indicated by the association of hypogonadism with the autoimmune polyglandular syndromes, which are caused by genetic defects in $CD4^+CD25^+$ Treg cell development and in the autoimmune regulator (Aire) gene that regulates thymic expression of various tissue-specific autoantigens, particularly autoantigens of the endocrine system (Ramsey et al. 2002; Kriegel et al. 2004). However, this hypogonadism is primarily due to autoimmunity against the steroidogenic (Leydig) cells within the testicular interstitium, and the damage to spermatogenesis and sperm production may be a secondary effect (Maclaren et al. 2001). In the Aire-deficient mouse, testis function appears to be normal, but sperm antibodies develop, leading to loss of fertility, and the epididymis contains numerous inflammatory infiltrates (Hubert et al. 2009). In several strains of rats and mice, thymectomy at 3 days of age, which causes a reduction in regulatory T cell subsets in the adult, results in spontaneous epididymo-vasitis and, eventually, orchitis (Lipscomb et al. 1979; Taguchi and Nishizuka 1981; Tung et al. 1987a). Studies in mice, rats, and rabbits have shown that tolerance to testicular antigens, leading to orchitis, epididymitis, and vasitis, can also be broken by active immunization with testicular or sperm homogenates or, more significantly, by passive transfer of T cells from actively immunized animals (Tung et al. 1971; Tung and Fritz 1984; Tung et al. 1987b; Mahi-Brown and Tung 1989). In some strains of mice, rats, and rabbits, autoimmune epididymo-orchitis may also develop following vasectomy (Alexander and Tung 1977; Taguchi and Nishizuka 1981; Flickinger et al. 1990), and there is some evidence that this may also occur in a small subset of human vasectomies (Goldacre et al. 2007). Overall, the evidence suggests that active tolerance is involved in protecting critical testis autoantigens and, apparently, in protecting sperm autoantigens in the rest of the reproductive tract (epididymis and vas deferens), thereby contributing to immune privilege in the testis.

On the other hand, studies on graft rejection responses in the rat and mouse testis suggest that immune privilege may also involve a failure to recognize antigens or to subsequently activate immunity when these antigens are first encountered within the testis environment. It has been shown that even long-standing intratesticular allografts are rapidly rejected when the host is sensitized to the graft antigens external to the testis, either by active immunization or by grafts of donor tissue to the skin, for example (Head et al. 1983a; Head and Billingham 1985a). This is consistent with the fact that both active immunization and passive transfer of activated lymphocytes from immunized animals to naïve recipients can cause testicular antibody formation and autoimmune orchitis (Tung et al. 1987b; Mahi-Brown and Tung 1989).

Experimental evidence that the testis is able to specifically regulate immunity comes from studies showing that introduction of antigens via the testicular route is able to induce suppression of T cell-mediated immunity against the injected

antigens. For example, prior injection of the relevant dominant antigens into the testis is able to ameliorate the experimental induction of autoimmune uveoretinitis, adjuvant-induced arthritis, or autoimmune encephalomyelitis (Li et al. 1997; Ditzian-Kadanoff 1999; Veräjänkorva et al. 2002). The mechanisms underlying this acquired immune deviation (AID) have not been studied in any detail in the testis, but they are probably analogous to mechanisms that operate in other well-characterized immunologically privileged sites, such as the eye and brain. Immune privilege in these tissues have been shown to involve the localized production of immunosuppressive cytokines, such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), and induction of antigen-specific immunoregulatory lymphocytes, specifically Treg cells, natural killer (NK) T cells, and γδT cells (Wilbanks et al. 1992; Sonoda and Stein-Streilein 2002; Ashour and Niederkorn 2006). Studies on pancreatic islet allografts in the mouse testis have shown that activated and memory T cells directed against graft antigens are selectively deleted within the testicular environment and that graft antigen-specific Treg cells are preferentially induced (Dai et al. 2005; Nasr et al. 2005). Moreover, NK cells, NKT cells, and Treg cells are strongly represented among the lymphocytes found within the interstitial tissue of the rat and mouse testis even under normal conditions (Tompkins et al. 1998; Jacobo et al. 2009) (Hedger, unpublished data). These data suggest that antigen-specific immunity within the testicular environment tends to favor an immunoregulatory or tolerogenic response, rather than a cell-mediated immune response that would normally be associated with graft rejection and autoimmunity.

Another characteristic feature of the testis relevant to immunity is the large population of resident macrophages within the interstitial tissue (Fig. 1) (Hedger 2002). The macrophages tend to be closely associated with the Leydig cells, with which they maintain intimate structural and functional interactions (Miller et al. 1983; Raburn et al. 1993; Wang et al. 1994; Gaytan et al. 1996). Much smaller numbers of dendritic cells are also present (Itoh et al. 1995; Fijak et al. 2005; Rival et al. 2006). The testicular macrophages and dendritic cells express major histocompatibility complex (MHC) class II antigens, indicating their capacity to interact with and activate $CD4^+$ T cells, although there is evidence that this expression may be impaired to some extent (el-Demiry et al. 1987; Tung et al. 1987b; Wang et al. 1994). In studies from the rat and the mouse, the majority of macrophages have been shown to be alternatively activated, with greatly reduced capacity for lymphocyte activation and proinflammatory cytokine production, and enhanced production of immunoregulatory cytokines, most notably IL-10 (Kern et al. 1995; Bryniarski et al. 2004; Winnall et al. 2011). Given that macrophages are the pivotal cells in the initiation of inflammation and subsequent immune responses, the functional properties of the testicular macrophages are entirely consistent with the manifestations of testicular AID. The functional activity of the dendritic cells of the testis remains to be examined in detail, but existing data suggest that they are immature and, therefore, will tend to be tolerogenic (Rival et al. 2007).

In summary of this section, all the existing data point to the conclusion that immune responses within the testicular environment tend toward suppression of

antigen-specific cell-mediated responses, favoring tolerogenic immune responses instead. On balance, this indicates that testicular immune privilege is actively maintained and regulated.

6 The Testicular Environment and Immune Privilege in the Testis

There are two widely held misconceptions about immune privilege as it pertains to the testis. The first misconception is that the testis has deficient or abnormal lymphatics and that restrictions on the movement of immune cells and other effectors contribute to an absence of immune responses within the testis. In fact, it has been clearly established in several species, including the experimental rodents used to study immune privilege, that the testis possesses effective lymphatics draining directly to local lymph nodes (Fawcett et al. 1973; Moller 1980; Head et al. 1983b; Itoh et al. 1998). Macrophages, dendritic cells, and lymphocytes, and even eosinophils and mast cells (Anton et al. 1998), are found throughout the testicular interstitium, which encompasses the intratesticular lymphatic vessels and is the site where surviving intratesticular grafts are normally located. Moreover, both MHC class I and class II antigens are expressed throughout the interstitial tissue (el-Demiry et al. 1987; Haas et al. 1988; Pöllänen et al. 1992; Wang et al. 1994), and antigens introduced into the testis interstitium can induce antigen-specific T cell responses (Head et al. 1983b; Ditzian-Kadanoff 1999; Nasr et al. 2005). As a corollary, it should be noted that the significantly lower temperature of the testis in most mammalian species does not appear to contribute to immune privilege (Selawry and Whittington 1984; Head and Billingham 1985a).

The second misconception is the role of the blood-testis barrier. In contrast to the blood-brain barrier, this barrier is not located at the level of the vascular endothelium of the testis. In the testis, the vascular endothelium is comparatively permeable to circulating immune cells, as already noted, as well as immune effector molecules, such as circulating cytokines, immunoglobulin, and complement (Yule et al. 1988; Hedger and Hettiarachchi 1994; Pöllänen et al. 1995; McLay et al. 1997). The blood-testis barrier is actually created by highly specialized tight junctions between the adjacent Sertoli cells, which serve to effectively separate the seminiferous epithelium into an adluminal and a basal compartment (Fig. 2) (Setchell et al. 1969; Dym and Fawcett 1970; Meng et al. 2005). The adluminal compartment of the seminiferous epithelium, where the majority of the developing spermatogenic cells and their unique autoantigens are located, constitutes a highly specialized biochemical environment that is also characterized by reduced MHC expression and the absence of immune cells, immunoglobulin, and other immune effector molecules (el-Demiry et al. 1987; Haas et al. 1988; Pöllänen et al. 1992). This contributes to reduced immunological activity against spermatogenic cells in the adluminal compartment, and disruption of the blood-testis barrier is a critical

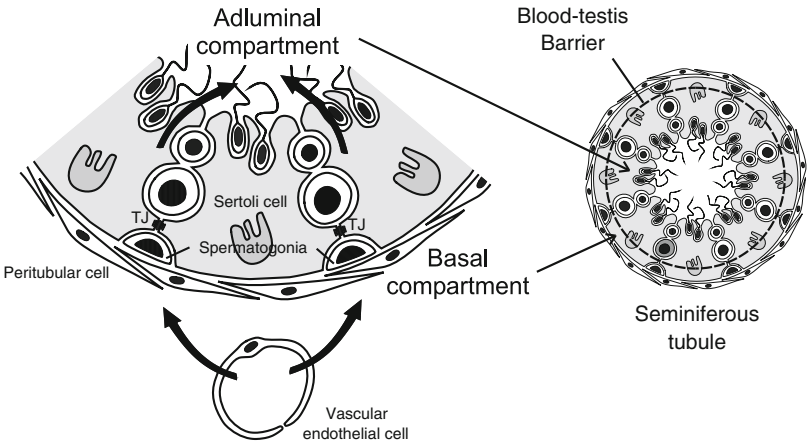

Fig. 2 The location and structural features of the blood-testis barrier. The blood-testis barrier is created by highly specialized occluding tight junctions (TJ) located between adjacent Sertoli cells in the basal region of the seminiferous epithelium. These junctions entirely obstruct the space between the Sertoli cells and effectively separate the tubule environment into a basal compartment (containing the spermatogonia and some early spermatocytes), the composition of which is largely determined by the circulation (*arrows*) and interstitial tissue products, and the adluminal compartment (containing the spermatocytes, spermatids, and spermatozoa), the composition of which is determined almost exclusively by secretions of the Sertoli cells (*arrows*). Immune cells and immune effector macromolecules, including complement, antibody, and cytokines, are normally unable to traverse this barrier

step in the onset of autoimmune orchitis and infertility (Kohno et al. 1983; Yule and Tung 1993; Itoh et al. 2005; Meng et al. 2011). Nonetheless, immunogenic sperm autoantigens are also found on spermatogenic cells located in the basal compartment of the epithelium without provoking immunity (Yule et al. 1988), and recurrent disruption of the blood-testis barrier, as occurs in seasonally reproducing animals, does not typically cause overt autoimmunity (Pelletier 1986). Moreover, the functional blood-testis barrier is limited to the epithelium of the seminiferous tubules—the epithelium of the straight tubules linking the seminiferous tubules to the rete testis and the rete testis itself lacks the specialized tight junctions found between adjacent Sertoli cells and appears to exhibit only the typical tight junctions associated with mucosal epithelia (Osman and Plöen 1978; Ghabriel et al. 2002). Accordingly, these epithelia tend to be more permeable to immunoglobulin and to lymphocytes (Koskimies et al. 1971; Dym and Romrell 1975; Knee et al. 2005; Naito and Itoh 2008) and represent the initial sites of orchitis in the activated lymphocyte transfer model of autoimmune orchitis in mice (Tung et al. 1987b; Yule and Tung 1993).

The preceding observations regarding the testicular lymphatics and blood-testis barrier further highlight the fact that immune privilege is an active process maintained by the testis, rather than a simple property of structural elements that

might restrict the movement of immune cells and other effectors within the testis. Furthermore, it is the somatic cells of the testis that have been most directly implicated in regulating testicular immune privilege.

Regulation of the testicular macrophage population has been extensively studied in both rats and mice. These studies have shown that recruitment and maintenance of the resident testicular macrophage population is controlled by the Leydig cells, although this does not appear to involve testosterone (Raburn et al. 1993; Duckett et al. 1997b; Meinhardt et al. 1998). The recruitment is gonadotropin regulated, as removal of LH by various means also causes macrophage numbers to decline, suggesting that this is a function of the mature Leydig cell (Gaytan et al. 1994; Duckett et al. 1997a; Duckett et al. 1997b). In fact, testicular macrophage numbers increase rapidly during puberty, in parallel with the increase in mature Leydig cell numbers at this time (Hardy et al. 1989; Ariyaratne and Mendis-Handagama 2000). The actual mechanisms involved are still unclear, but in rats and mice, these two cell types are physically connected, forming clusters with highly specialized interdigitations between them (Miller et al. 1983; Hutson 1992). On the other hand, evidence suggests that the function of the testicular macrophages is regulated by the Sertoli cells, involving FSH-dependent mechanisms (Duckett et al. 1997a). Thus existing data suggest that recruitment of macrophages and their unique phenotype are functions of the mature testis under indirect gonadotropin regulation, through both the Leydig cells and the Sertoli cells (Fig. 3). In turn, the number of lymphocytes appearing within the testicular interstitial tissue is related to the size and activity of the macrophage population (Wang et al. 1994; Hedger et al. 1998; Hedger and Meinhardt 2000). Surprisingly, the presence of developing spermatogenic cells, which are also dependent upon the activity of the Leydig and Sertoli cells, does not appear to be essential either for maintenance of the testicular macrophage population (Meinhardt et al. 1998) or for persistence of testicular immune privilege defined by intratesticular graft survival (Selawry and Whittington 1984; Head and Billingham 1985a; Whitmore et al. 1985).

While the effects that the Leydig cell exerts on immune cell activity in the testis appear to be largely indirect, the Sertoli cell appears to play a much more immediate role in controlling intratesticular immunity. Not only do these cells have inherent immunosuppressive activity in lymphocyte cultures (Wyatt et al. 1988; Selawry et al. 1991; De Cesaris et al. 1992), they also show unique favor as transplants and are even able to provide protection for allogeneic and xenogeneic grafts of other cell type transplanted along with them (Selawry and Cameron 1993; Sanberg et al. 1996; Suarez-Pinzon et al. 2000). This protection does not depend upon the formation of tight junctions or barriers and appears to be due to properties inherent to the Sertoli cell itself (Mital et al. 2010). A number of immunoregulatory proteins produced by these cells have been identified: inhibitors of complement and granzyme B activity (O'Bryan et al. 1990; Sipione et al. 2006; Lee et al. 2007); lymphocyte-inhibiting molecules, such as Fas ligand (CD95L)(Bellgrau et al. 1995; Sanberg et al. 1997), indoleamine 2,3-dioxygenase (Fallarino et al. 2009), nonclassical MHC antigens (Slukvin et al. 1999; Ryan et al. 2002), and the inhibitory coreceptor B7-H1 (Dal Secco et al. 2008); and immunoregulatory cytokines, most

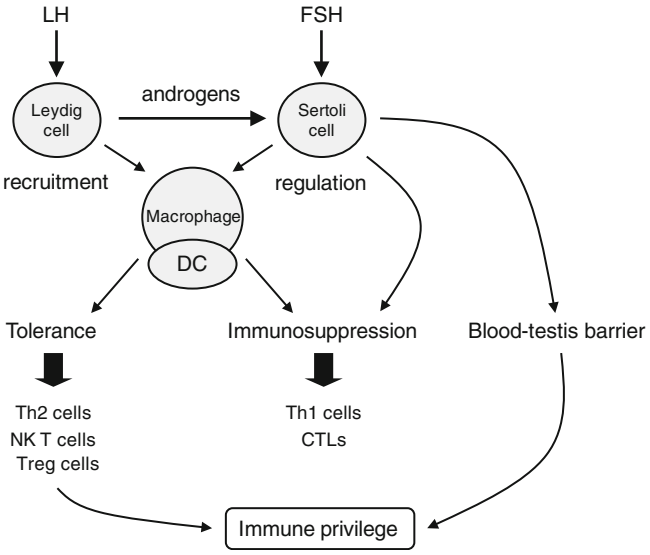

Fig. 3 Summary of the putative mechanisms controlling immune responses within the testis. The immunological environment of the testis is regulated by the gonadotropins secreted from the anterior pituitary: luteinizing hormone (LH), which acts on the Leydig cells, and follicle-stimulating hormone (FSH), which acts on the Sertoli cells. In response to stimulation by LH, the Leydig cells produce androgens, particularly testosterone, which are necessary to support mature Sertoli cell functions. These functions include the maintenance of the tight junctions that comprise the main structural elements of the blood-testis barrier. The Leydig cells are responsible for recruiting macrophages into the interstitial tissue. Under the influence of the testicular environment, chiefly mediated by the Sertoli cells, the resident macrophages adopt an alternatively activated phenotype, characterized by reduced proinflammatory activity and preferential production of immunoregulatory cytokines, such as IL-10. Dendritic cells (DC) within the testicular interstitium appear to be functionally immature. The Sertoli cells, resident macrophages, and dendritic cells regulate the development of lymphocytes circulating through the testis, promoting the activity of regulatory T cells (e.g., Th2 cells, NKT cells, and Treg cells) and suppressing the activity of Th1 cells and cytolytic T cells (CTLs). This results in active tolerance to antigens encountered within the testis (and its draining lymph nodes) and inactivation of antigen-specific effector cells directed against testicular antigens, culminating in immune privilege

notably TGF-β and activin A (Skinner and Moses 1989; Suarez-Pinzon et al. 2000; Okuma et al. 2005). The relative importance of each of these Sertoli cell products toward maintaining testicular immune privilege remains to be established, but identifying the critical mechanisms that confer the ability to locally suppress graft rejection responses on the Sertoli cell may have considerable impact upon transplantation medicine.

Lipidic molecules with immunoregulatory or immunosuppressive activity, including testosterone, several of the eicosanoids, and some lysophosphatidylcholines, are produced by various testicular cell types. Testosterone, as well as other androgens, produced by the Leydig cells have immunosuppressive effects on macrophage and lymphocyte activity that are believed to contribute to

gender-discordant immune functions, such as the reduced susceptibility to certain autoimmune diseases in men and differences in innate and adaptive immunity between the sexes (Grossman 1985; Wichmann et al. 1997; Rettew et al. 2008). Testosterone concentrations within the testis are more than an order of magnitude higher than in the circulation or in other tissues and are also elevated in the epididymis (Jean-Faucher et al. 1985; Turner et al. 1985). Although early studies were equivocal concerning the relationship between intratesticular androgens and graft survival in the testis (Whitmore and Gittes 1978; Head and Billingham 1985a; Selawry and Whittington 1988; Cameron et al. 1990), there is evidence that androgens inhibit the progression of autoimmune orchitis in experimental models of the disease (Fijak et al. 2011). Furthermore, specific deletion of the androgen receptor on the Sertoli cell leads to disruption of the blood-testis barrier and an increase in intratesticular leukocytes and antibodies against spermatogenic cell antigens (Meng et al. 2011). Conversely, estrogens appear to promote intratesticular inflammation and have an inhibitory effect on graft survival in the testis (Head and Billingham 1985a; Li et al. 2006). Eicosanoid biosynthesis, which occurs in most testicular cell types, leads to production of several immunoregulatory and anti-inflammatory molecules, particularly prostaglandins E and D (Sorrentino et al. 1998; Winnall et al. 2007), although their importance in regulating immune responses in the testis has yet to be established. The presence of several medium chain-length lysophosphatidylcholines, which are produced during eicosanoid biosynthesis and several other processes of phospholipid metabolism, in the fluid of the testicular interstitial tissue has been shown to be responsible for the profoundly suppressive effect of these fluids on lymphocyte activity in vitro (Foulds et al. 2008).

Altogether, it appears that immune privilege in the testis, and possibly in the rest of the male reproductive tract, is maintained by a unique testicular environment that controls immune cell activity, inducing and maintaining peripheral tolerance and suppressing adaptive immunity in a tissue-localized manner (Fig. 3). This regulation involves specific hormone-dependent actions of the unique somatic cells of the testis, the Sertoli and Leydig cells, rather than simple sequestration of antigens or restriction of immune cell access.

7 The Consequences of Testicular Immune Privilege

The existence of immune privilege has important implications for both normal testicular function and male reproductive disease (Fig. 4). Although the testis is a common site for relapsing leukemia following therapy, which may be attributable to its unique immunological environment (Hudson et al. 1985; Kim et al. 1986), the testis does not display an increased susceptibility to tumors or infections compared with other tissues. In fact, infections of the testis are relatively rare in comparison with more distal tissues of the male reproductive tract (Krieger 1984). The intensity of inflammatory responses in the testis may be reduced, due to the unique regulatory

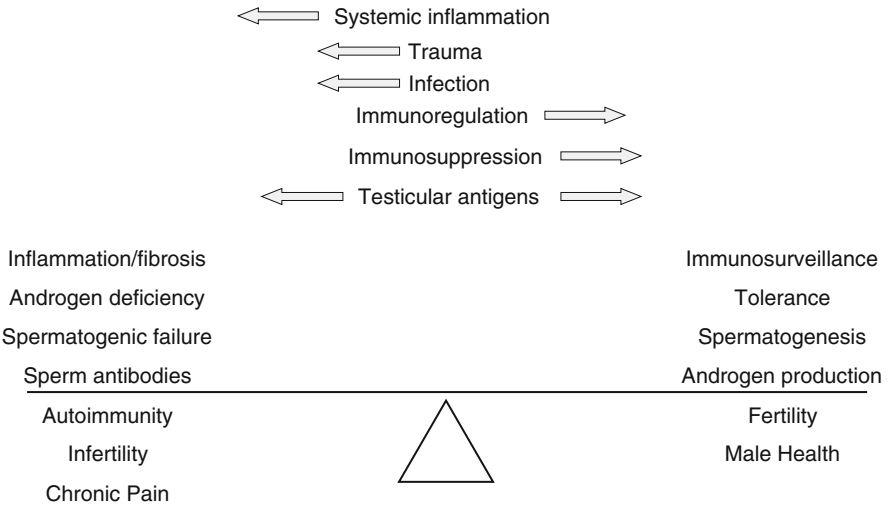

Fig. 4 The balance between disease and normal function in the testis. Testicular function is dependent upon a precise balancing act involving the immune system, maintained by testis-specific immunoregulatory processes. The manifestation of this control is immune privilege with respect to testicular autoantigens, which may be extended to foreign antigens as well, leading to extended graft survival. The balance may be tilted toward reproductive disease by genetic predisposition, together with precipitating events, such as systemic inflammation, reproductive tract trauma, or infection

properties of the testicular macrophages, and antigen-specific immunity may be compromised, but it appears that the ability of the testis to resist and clear infections is intact nonetheless. This may be due to an increased reliance on innate immunity, and there has been a steady increase in interest in the role of innate immunity in testicular function recently (Com et al. 2003; Bhushan et al. 2008; Starace et al. 2008).

Less intuitive is the fact that immune cells, cytokines, and other immune mediators appear to play a crucial role in normal testicular function. These include a role for the testicular macrophages in regulating Leydig cell proliferation, development, and steroidogenesis and the involvement of inflammatory signaling pathways and cytokines produced by the Sertoli cells in the control of spermatogenic cell development—these topics have been extensively reviewed elsewhere (Hedger and Hales 2006; Hedger 2011). This close relationship between innate immunity in the testis and normal testis function has consequences for fertility and provides an explanation for the observation that steroidogenesis and sperm production can be compromised by systemic infections and inflammatory disease (Dong et al. 1992; Baker 1998).

Finally, as previously mentioned, autoimmune infertility leading to testicular damage appears to be a problem for a small subset of men. Presumably, this is due to underlying genetics of susceptibility and is associated with a precipitating event, such as trauma to the reproductive tract or infection. The ability to predict, prevent,

and/or treat this condition will depend upon much better understanding of the unique immunobiology of the testis. It is increasingly evident that failure of these testicular mechanisms underlies male infertility, chronic inflammatory disease, and reproductive tract pain. More generally, uncovering the details of these mechanisms may lead to greater understanding of systemic immunity and tolerance in peripheral tissues and, potentially, for the development of novel immunosuppressive therapeutics.

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The Role of Intrauterine Immune Privilege in Perinatal Infectious Diseases

Takeshi Nagamatsu and Danny J. Schust

Abstract The pregnant uterus provides an immunologically "privileged" environment in which a genetically mismatched fetus typically thrives without deleterious allogeneic immune response. The mechanisms by which the fetus is protected from maternal immune attack are still incompletely understood but are undoubtedly multifactorial. Simple evasion of immune detection is no longer considered to be involved. There is now good evidence that the maternal immune system recognizes and responds to the presence of the fetus. However, maternal cellular and soluble immune responses, and those of the immunologically active, fetally derived trophoblast cells, generally support rather than harm the pregnancy. Gestational hormones and placenta-derived substances actively modulate maternal immunity and promote tolerance of the fetus. Still, the maternal immune system must continue to serve its protective role against pathogen invasion during pregnancy. Dramatic, pregnancy-related shifts in systemic and local immune responsiveness in the mother could compromise her ability to control infectious insults. To this point, clinical data indicate that pregnant women are more susceptible to specific types of viruses and parasites than their nonpregnant female counterparts. In this chapter, we will review the molecular mechanisms involved in creating the immunologically privileged intrauterine environment during pregnancy and discuss its costs to mother and fetus in terms of increased infectious disease risk.

Keywords Placenta • Chorioamnionitis • Trophoblast • Maternal-fetal interface • Tolerance

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1 Introduction

Over the course of evolution, vertebrate species have been endowed with the ability to rigorously recognize self and nonself. This allowed for intricate discrimination of self from allogeneic and altered syngeneic tissues and has been fundamental in the development of protective immunity against xenogeneic infectious organisms (Cooper and Alder 2006; Hirose 2003; Lightner et al. 2008). In this context, the reproductive process of viviparity in vertebrates is seemingly paradoxical, since a genetically mismatched fetus can grow in close contact with the tissues of an organ that resides in an immunocompetent maternal host. This organ, the maternal uterus, exhibits the remarkable ability to secure an "immunologically privileged" environment for the developing fetus that is essential to pregnancy success. Sir Peter Medawar first proposed the immunological conundrum of the fetal allograft well over a half century ago (Medawar 1961), and numerous studies have been conducted over the intervening years to clarify the mechanisms that protect the allogeneic fetus from maternal immune attack. While great progress has been made in this challenging field, a single overarching solution to this immunological dilemma has not been reached. Rather, the collective data point to an intricate and redundant series of maternal changes that integrate to create the remarkable immune environment within the gravid uterus. Placental trophoblast cells play an important role in the immune interactions between mother and fetus. Derived from extraembryonic stem cells, trophoblast cells form a structural barrier between the embryo and the maternal blood and tissues and are the only cells that directly confront maternal immune cells. Trophoblast cells help to secure the safety of the allogeneic fetus through restricted transplantation antigen (human leukocyte antigen, HLA) expression patterns and the production of immunomodulatory mediators (Blaschitz et al. 2001; Bulmer and Johnson 1985). The maternal host offers protection to her growing child by altering the effector characteristics of the immune cells populating the uterine decidua (the specialized endometrial tissue lining the uterus during pregnancy) so that they possess a dominant immunosuppressive phenotype. Further, a generalized decrease in the ability of maternal antigen-presenting cells to effect cytotoxic responses combines with a hormonal milieu-driven immune suppression to aid in the establishment of tolerance to fetal antigens.

These essential changes come at a price. The same local and systemic changes in the maternal immune system that favor pregnancy maintenance may put the mother at risk for pathogen invasion, resulting in maternal systemic infection, uteroplacental infection and/or fetal infection (Vargas-Villavicencio et al. 2009). Pregnant women are more likely than nonpregnant women to acquire a variety of viral and parasitic infections. Once infected, pregnant women are also more likely to endure systemic spread of these pathogens and to suffer serious infectious sequelae. More localized infection of the uterus and placenta (chorioamnionitis) is a major cause of pregnancy-related maternal and fetal morbidity and mortality, including preterm labor and preterm birth. In most cases of chorioamnionitis-related preterm

labor, the infection began several weeks prior to the onset of labor. The decidual lymphocytes that have been primed to protect the allogeneic fetus may fail to fully recognize pathogen entry, resulting in silent progression of infection, preterm rupture of membranes, and uncontrollable uterine contractions (Andrews et al. 2000). Chorioamnionitis can also progress to fetal infection. Since the fetal immune system does not complete its development until well after the birth of the child, the fetus is particularly vulnerable to transplacental pathogen attack, and congenital infections are frequently associated with serious irreversible damage in fetal organs or with fetal demise.

Although trophoblast cells function as an anatomical barrier to invasion by exogenous pathogens, some parasites and viruses have specific molecular mechanisms that allow them to target trophoblast cells as their primary host. Trophoblast cells with restricted HLA expression are resistant to $CD8^+$ T cell- and NK cell-mediated cytolysis. This trophoblast-specific characteristic could abrogate the typical effective mechanisms for immune surveillance that exist in the nonpregnant women and, instead, allow for unfettered transplacental transmission of toxoplasma and viruses such as HIV and cytomegalovirus (Al-Husaini 2008; Ortiz-Alegría et al. 2010; Syggelou et al. 2010). Some infectious organisms display molecules on their cell surface that specifically target chemokine receptors and adhesion molecules expressed on trophoblast cells. Others exploit pregnancy-specific changes in the physical barrier between mother and fetus to gain access to the immunologically vulnerable feto-placental unit.

In this chapter, we will address the unique immunological attributes and challenges at the maternal-fetal interface. We will begin by discussing the mechanisms involved in maternal immune tolerance of the fetal allograft through the creation of an immune-privileged intrauterine environment. We will finish by discussing how this same unique feto-protective intrauterine environment offers challenges to the pregnant mother when fighting particular infections, including bacterial chorioamnionitis, cytomegaloviral infections, toxoplasmosis, leishmaniasis, and malaria.

2 Maternal Immune Cells in the Decidua

In response to the combined influence of estrogen and progesterone, the uterine endometrium undergoes dramatic morphologic and functional changes after ovulation. These maturational changes prepare the endometrium, now called the uterine decidua, to accept a fertilized egg and to support placental development (placentation). Approximately one-half of the cells in the uterine decidua during early pregnancy are maternal hematopoietic cells, and this number decreases over the remainder of pregnancy. This abundant immune cell population is comprised of decidual natural killer cells (dNKs, 70%), T cells (10%), myelomonocytic cells (20%), and a very small number of B cells (King 2000). This differs dramatically from circulating maternal leukocyte populations, which contain many B and T cells

but very few NK cells. The unique leukocyte composition within the decidua and the altered biological properties of its constituents (discussed below), have been thought to be central to the promotion of maternal immune tolerance of the allogeneic fetus.

3 Major Histocompatibility Complex (MHC) Expression on Trophoblast Cells

Trophoblast cells are the only fetally derived cells to directly contact maternal immune cells. In this regard, pregnancy differs from organ transplantation, in which recipient cells encounter a variety of donor cells. The implanting blastocyst is comprised of an inner cell mass that will eventually develop into the fetus and an outer shell of trophoblast cells that will develop into the placenta. These trophoblast cells are the first fetally derived cells to contact the maternal decidua. As the embryo implants, the leading edge of trophoblast cells invades into the maternal tissue. After the embryo has become completely embedded in maternal tissue, it remains surrounded by trophoblast cell derivatives so that these are the only cells in direct contact with maternal tissues. As the placenta grows, human trophoblast-derived cells differentiate into two distinct subpopulations called syncytiotrophoblast (ST) and extravillous trophoblast cells (EVTs; Fig. 1). Both cell types originate from common stem cell-like trophoblast cells, called cytotrophoblasts (CTs) (Aplin 1991). The fetal side of the human placenta consists of a large number of branched structures called placental villi. Each villus contains fetal vessels surrounded by stroma. During early human pregnancy, these villi are completely covered by at least two layers of trophoblast cells. ST forms the outmost surface of the villous tree. Floating villi extend into the placental intervillous space and are bathed in maternal blood supplied from the uterine spiral arteries. Active turnover of ST results from continuous proliferation of CTs which line the inner side of ST layer covering the villi. Anchoring villi traverse the intervillous space to contact the maternal decidua. At the peripheral tip of the anchoring villi, where it attaches to the decidua, proliferating CTs differentiate into a third type of trophoblast cells, termed EVTs. EVTs block the flow of maternal blood through the spiral arteries and into the intervillous space until late in the first trimester of pregnancy. EVTs also leave the tips of the anchoring villi to invade deeply into maternal tissues, crossing the decidua and moving through the inner third of the myometrium. EVTs also play an important role in the reconstruction of the uterine vessels, replacing the vasoactive endothelium of the spiral arteries to create a vascular structure that is dilated and minimally responsive to maternal vasoactive substances. This assures preferential blood flow to the placental intervillous space when the trophoblast plugs dislodge after approximately 9–10 weeks of gestation (Kaufmann et al. 2003; Zhou et al. 2002).

While villous stromal cells derived from the inner cell mass of the blastocyst express MHC classes I and II, the ST layer is completely devoid of MHC expression at their cell surface (Sunderland et al. 1981). Since these cells are in direct contact

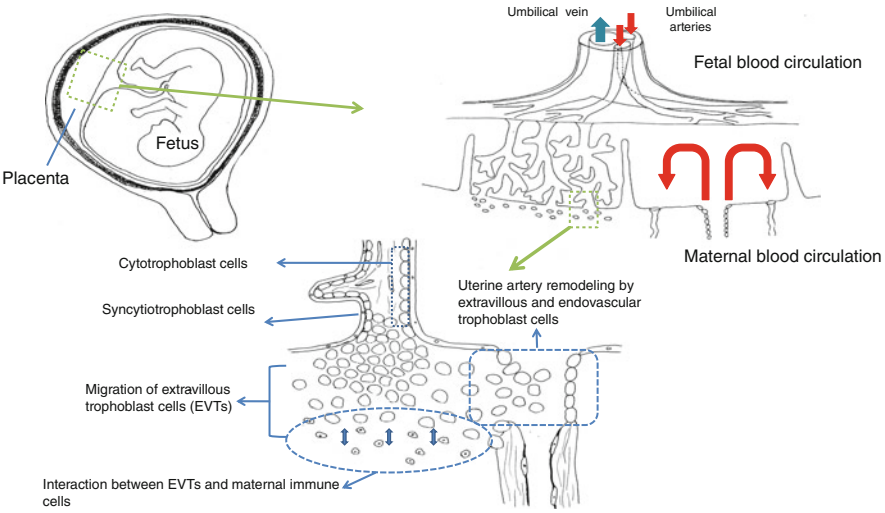

Fig. 1 Macroscopic and microscopic structure of the human placenta. The fetus resides in a fluid-filled amniotic sac, surrounded by uterine decidua within the gravid uterus. The umbilical cord brings blood to the fetus through the single umbilical vein and from the fetus through paired umbilical arteries. Within the body of the placenta, fetal vessels are surrounded by placental mesenchyme and by two layers of trophoblast cells in structures called placental villi. The inner, cytotrophoblast cell layer consists of stem cell-like placental cells that give rise to the outer layer of syncytiotrophoblast cells and to those cytotrophoblast cells that differentiate into extravillous and endovascular cytotrophoblast cells. Extravillous cytotrophoblast cells (EVTs) invade through the uterine decidua and into the inner third of the uterine myometrium. They aid in remodeling the distal maternal spiral arteries externally. Endovascular trophoblast cells remodel the distal spiral arteries internally. Both eventually replace the walls of the maternal arteries to create low-resistance, poorly vasoactive conduits that transport blood from mother to the placental intervillous space

with the maternal blood in the intervillous space, this lack of antigenicity may partially explain escape from maternal allogeneic immune responses. Unlike ST, EVTs express MHC class Ib molecules, including HLA-G and HLA-E. They also express the MHC class Ia molecule, HLA-C, but not HLA-A or HLA-B (Ishitani and Geraghty 1992; Ishitani et al. 2003; King 2000; King et al. 1996; Kovats et al. 1990). Common features of HLA-C, HLA-E, and HLA-G include a relatively low level of expression at the cell surface, limited polymorphism at peptide binding sites, and a reduced potential to activate T cell-mediated cytotoxicity (Furman et al. 2000). This helps EVT to avoid harmful alloreactions. Like trophoblast cells in the human placenta, murine trophoblast cells do not express class II antigen-presenting molecules (Hedley et al. 1989; Redline and Lu 1989; Zuckermann and Head 1986). Murine labyrinthine trophoblast cells (Fig. 2) functionally mimic human syncytiotrophoblast cells and, along with murine giant trophoblast cells, lack MHC class I expression. Trophoblast cells in the murine spongiotrophoblast layer (including glycogen cells) lie in direct contact with maternal cells. Although some spongiotrophoblast cells are reported to express MHC class I, the expression levels are far lower than that in the spleen of the adult mouse (Billington and Burrows 1986; Redline and Lu 1989).

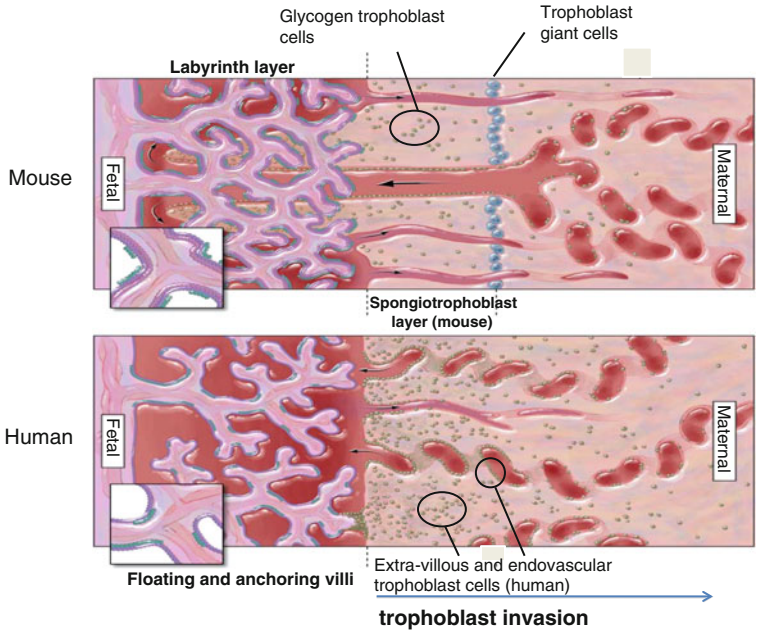

Fig. 2 Comparison of murine and human placental structures**.** Despite many structural similarities, several important differences distinguish the murine and human placentas. The villous structures of the human placenta are branching and tree-like while those of the mouse are labyrinthine. Invasive trophoblast cells in the human include extravillous and endovascular trophoblast cells; those of the mouse include glycogen trophoblast cells and trophoblast giant cells. Trophoblast invasion into the maternal decidua is significantly more extensive in the human ()

Trophoblast MHC expression patterns that are characterized by restricted MHC subtypes and highly limited localization may be an important strategy for fetal evasion of allogeneic T cell responses. Trophoblast cells are, in fact, resistant to lysis by $CD8^+$ T cells in vitro while fetal fibroblasts are not (Zuckermann and Head 1987). However, this unique pattern of MHC expression is certainly only one way by which the fetus is protected from allogeneic T cell responses, and the true importance of trophoblast expression patterns is likely obfuscated by redundancy in protection mechanisms. To this point, allogeneic pregnancies are unaffected when maternal T cells are induced to express a T cell receptor (TCR) which specifically recognizes paternal MHC class I in transgenic murine models (Tafuri et al. 1995). Fetal growth and survival was maintained in these animals even if trophoblast expression of paternal MHC was enhanced by transgenic injection of plasmid containing paternal MHC products and promoter elements from HLA-G and placental lactogen (Rogers et al. 1998; Zhou and Mellor 1998). In some of these TCR transgenesis investigations, the number of T cells reactive to fetal allogeneic H2 antigen (mouse MHC) was reduced in the spleen and the maternal lymph nodes during pregnancy, suggesting that pregnancy has suppressive systemic effects on alloreactivity (Jiang and Vacchio 1998; Tafuri et al. 1995). Other investigators have

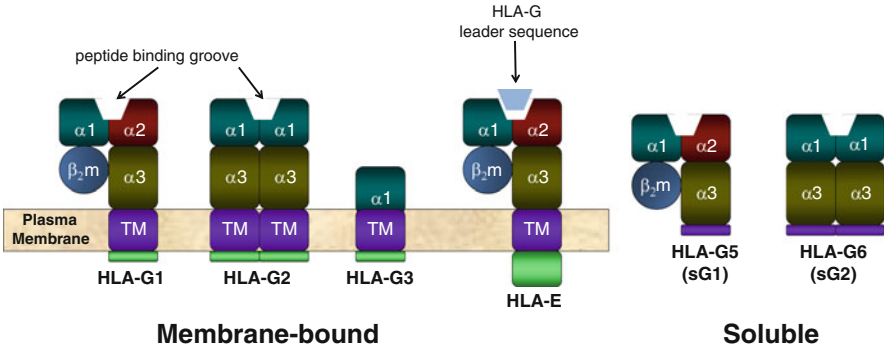

Fig. 3 Structure of the MHC class Ib products, HLA-G and HLA-E. When stably expressed at the cell surface, MHC class Ia and Ib products typically exist as trimers of MHC class I heavy chain, an invariant chain, β_2m, and an 8–10 amino acid peptide fragment bound in the peptide-binding groove. The peptide-binding groove of the MHC class I heavy chain is formed by segments of the α1 and α2 domains. The remainder of the class I heavy chain includes an α3 domain, a transmembrane segment and a C-terminal cytoplasmic tail. HLA-G differs from most other MHC class I molecules in that it has a shortened cytoplasmic tail and can exist as several spliced variants. Some of these variants lack their transmembrane segments, making them soluble. The heavy chain of HLA-E has a full-length cytoplasmic tail, and its peptide-binding grove commonly binds leader sequences from other class I heavy chains

reported a contradictory observation, demonstrating an expansion in H2-reactive maternal $CD8^+$ T cells in the lymph nodes draining the uterus (Zhou and Mellor 1998). Although an explanation for these discrepancies has not been put forth, these studies seem to indicate that restricted trophoblast MHC expression is not necessarily a prerequisite for the maintenance of pregnancy. Other regulators of maternal T cell response to fetal antigen are likely involved.

4 HLA-G

Because of its unique expression pattern (limited to the EVT, the thymus, and the fetal testis), the MHC class Ib molecule HLA-G has received particular attention in the field of reproductive immunology. Transfection of HLA-G into target cells has been shown to decrease antigen-specific cytotoxic T cell response to and NK cell-mediated lysis of these cells (Khalil-Daher et al. 1999; Le Gal et al. 1999). HLA-G binds to the leukocyte immunoglobulin-like receptors (LILR) B1 and LILRB2. These receptors are expressed on a variety of leukocyte subpopulations, and their downstream post-ligation signaling typically results in immune inhibitory effects. HLA-G exists in several spliced variant forms in vivo (Fig. 3). A common soluble isoform of HLA-G (HLA-G5) is translated from a truncated mRNA resulting from a stop codon in intron 4 and lacks its transmembrane segment and cytoplasmic tail (Fujii et al. 1994). Soluble HLA-G is produced by all human trophoblast subtypes, including ST and EVT. It suppresses Th1 cytokine secretion and protects the Th2

cytokine milieu favorable for pregnancy maintenance (Kanai et al. 2003). Despite mechanistic studies supporting an important role for HLA-G in immune modulation at the human maternal-fetal interface, homozygosity for a null allele of HLA-G gene does not affect human fertility or pregnancy outcomes (Ober et al. 1998). These data suggest the protective function of HLA-G during pregnancy may also be redundant.

5 Decidual NK Cells

Since large granular lymphocytes, classified as NK cells, are the dominant subset among the leukocytes populating the decidua during early pregnancy, the function of dNK cells has been a particularly intriguing target of study for reproductive immunologists. Decidual NK cells have several properties that distinguish them from their peripheral counterparts. When subclassified by cell surface markers, NK cells can be divided into those that are $CD56^{bright}CD16^{-}$ and those that are $CD56^{dim}CD16^{+}$. $CD56^{bright}CD16^{-}$ NK cells predominate in the decidua but are fairly rare in the periphery, whereas $CD56^{dim}CD16^{+}$ cells comprise 90% of the total NK cell population in the peripheral blood. There is an accumulating body of evidence showing that the primary role of dNK cells is not cytotoxicity but rather the support of placental development. This includes activities that are intimately involved in decidual maturation, uterine artery remodeling, and the control of trophoblast invasion (Hanna et al. 2006). Although dNK cells are fully equipped with cytotoxic molecules such as perforin and granzyme A (King et al. 1993), freshly isolated dNK cells exhibit diminished cytotoxic function against target cells in vitro when compared to their peripheral counterparts (Manaseki and Searle 1989). Interestingly, trophoblast cells are constitutively resistant to cell lysis not only by dNK cells but also by peripheral NK cells, unless the latter are activated with IL-2 and IL-12 (Hayakawa et al. 1999; King et al. 1990). The exact mechanism for trophoblast resistance to NK cytotoxicity remains unclear. What is known is that the signaling pathways for activation and inhibition of NK-mediated killing rely on the relative NK cell surface expression and ligation of receptors promoting and inhibiting cell-mediated cytotoxicity. These NK cell surface receptors include natural cytotoxicity receptors (NCRs) and MHC-dependent receptors, such as the killer immunoglobulin receptors (KIRs) and the heterodimer NKG2A/CD94. The expression patterns of these receptors on the dNK cell surface, when combined with the expression of their ligands on placental and uterine decidual cells, are central to our understanding of the regulation of NK cell activity at the maternal-fetal interface. The expression profile of KIRs on human dNK cells is skewed toward HLA-C recognition and parallels the selective expression of HLA-C on EVTs (Sharkey et al. 2008). A genetic analysis of maternal KIR haplotypes (KIR AA, AB, and BB) and matched maternal and fetal HLA-C genotypes revealed that women who carry the KIR AA haplotype, which results in the abrogation of the activating KIR2DS1 receptor, have an increased risk for recurrent pregnancy loss and preeclampsia. This is particularly true among women whose fetal genotype contains more genes for the KIR2DS1 ligand (group 2 HLA-C genes) than the

mother. Mothers who possessed the telomeric end of the KIR B region, which contains KIR2DS1, exhibited less frequent pregnancy disorders (Hiby et al. 2010; Hiby et al. 2008). These results strongly suggest that activation of dNK cells through paternal group 2 HLA-C promotes an immune environment that favors of pregnancy maintenance.

Receptors for HLA class Ib molecules have also been found on decidual NK cells. To allow stable HLA-E expression at the cell surface, the MHC class Ib molecule HLA-E often binds leader peptide sequences cleaved from other HLA class I products within its peptide-binding grove. These leader sequences are frequently derived from HLA-G molecules. NK cells recognize HLA-E combined with HLA class I leader sequences through binding to the heterodimeric inhibitory receptor NKG2A/CD94 or the activating receptor NKG2C/CD94 (Lee et al. 1998). Decidual NK cells show an increased level of inhibitory NKG2A molecule expression, but a similar expression of activating NKG2C when compared to matched peripheral NK cells (King et al. 2000; Kusumi et al. 2006). Expression of HLA-E coupled with an HLA-G-derived leader peptide at the trophoblast cell surface might enable EVTs to escape dangerous cytotoxic dNK cell activity through interactions with NKG2A/CD94. KIR2DL4 is expressed on all NK cells and binds specifically to HLA-G but not other HLA class I molecules (Rajagopalan and Long 1999). Following engagement with KIR2DL4, soluble HLA-G is endocytosed into Rab5-positive compartments via a dynamin-dependent process, facilitating the production of proinflammatory and proangiogenic cytokines by the NK cell (Rajagopalan et al. 2005). Although incompletely defined, there is mounting evidence that dNK cells and fetal trophoblast cells cooperate during placental development, particularly during the establishment of an appropriately functioning utero-placental vasculature. Recent investigations have linked the decidual NK cell production of multiple chemokines and cytokines, including IFN-γ, IL-8, SDF-1, IP-10, and VEGF, to healthy placental angiogenesis (Hanna et al. 2006; Hanna et al. 2003; Manaster et al. 2008).

6 Presentation of Fetal Antigens by Maternal APCs

The developing fetal allograft has been frequently compared to an allogeneic tissue transplant. While the fetus differs from a true allotransplant in that it shares transplantation antigens with the maternal host, the analogy retains some merit. As discussed, the abrogation of MHC class II molecule expression and restricted expression of MHC class I molecules on those trophoblast cells that contact maternal immune cells may be an important mechanism to minimize activation of maternal T cells by fetal APCs presenting allogeneic antigens. Organ transplants, however, are plagued by the induction of T cell activation not only by donor APCs but also by recipient APCs (Game and Lechler 2002). Recent evidence indicates that the fetal allograft promotes similar challenges. Maternal dendritic cells appear to present fetus-derived antigens, which results in allogeneic activation of maternal T cells. There are two possible mechanisms by which this indirect antigen recognition might occur. The first would involve the migration of decidual APCs loaded

with fetal antigen to the maternal lymph nodes. The second would invoke passive transport of soluble factors and fetal cell components into regional maternal lymph nodes where they would be presented to maternal T cells on the surface of resident maternal APCs. The former mechanism is unlikely in mouse pregnancy since murine lymphatic vessels invade the uterine myometrium but not the decidua (Collins et al. 2009). It has been shown that DCs in the murine decidua do not migrate from the decidua, even after potent activation upon exposure to lipopolysaccharide (LPS) (Collins et al. 2009). Less is known about the lymphatics in human decidua, although there is recent evidence that their architecture may mimic that seen in mice. In fact, one recent study demonstrated that the process of human decidualization causes a regression of lymphatic vessels, particularly in areas surrounding the spiral arteries (Volchek et al. 2010).

There exists much better evidence that antigens derived from the placenta and the fetus can reach maternal lymphatic organs. Fetal DNA can be detected in the maternal circulation as early as the 4th week of gestation (Thomas et al. 1995). The surfaces of placental villi are continually shedding apoptotic cellular debris as a part of their normal growth and development. Villous structures, known as syncytial knots, represent the site of this apoptotic process. These knots result from the turnover of syncytiotrophoblast layer and may be an important source of the fetal DNA detected in the maternal circulation during pregnancy. Entire fetus-derived cells can also be found in maternal blood and tissues, and these cells may remain detectable for many years after delivery (Bianchi et al. 1996). This common and interesting phenomenon has been called fetal-maternal microchimerism. The fetal cells involved in microchimerism are thought to be of hematopoietic and mesenchymal origin. Direct evidence for transport of fetal antigen into maternal lymphatic organs was generated in mice impregnated with Act-mOVA transgenic conceptuses that express a membrane-bound form of OVA driven by a ubiquitously active β-actin promoter. Immunohistochemistry demonstrated that ovalbumin shed from trophoblast cells was presented by follicular dendritic cells residing in the maternal spleen and lymph nodes. When anti-OVA TCR transgenic T cells (OT-I and OT-II cells) were transferred into pregnant mice with OVA-expressing fetuses, OVA-driven proliferation of adoptively transferred T cells was observed. Notably, the expansion of OVA-responsive T cells did not affect the resorption of OVA^+ fetuses, even when cytotoxicity was enhanced by combined administration of anti-CD40 Abs and poly(I:C), enhancers of APC functions (Erlebacher et al. 2007). In short, even though maternal T cells are indirectly exposed to fetal antigen in lymphatic organs, mechanisms exist to protect the fetus from the attack by sensitized maternal T cells.

7 Regulatory T Cells

Classic work using animal transplantation models demonstrated prolonged survival of fetal skin grafted onto a pregnant mother with rapid rejection at the completion of pregnancy; similar grafts were also promptly rejected by unrelated hosts

(Anderson 1965; Anderson and Benirschke 1964). Using mice engineered to constitutively express a TCR recognizing the H-2K^b antigen, mothers pregnant with Kb-positive conceptuses tolerated induction of a Kb-expressing tumor, while mice with syngeneic and third-party allogeneic conceptuses did not (Tafuri et al. 1995). These observations support the concept that pregnancy induces the development of immune cells with suppressor activities that might, in turn, promote tolerance specific to fetal antigens. Very recent progress in our understanding of autoimmunity and graft rejection has identified several specialized T cell subsets involved in central and peripheral immune tolerance. Among these tolerogenic T cell populations, CD4$^+$CD25$^+$ regulatory T cells (Treg) have been the most closely linked to fetomaternal immunity. Two murine Treg depletion models have been instrumental in advancing the field; the first employs anti-CD25 antibody-mediated Treg depletion in pregnant mice, while the second uses T cell-deficient pregnant nude mice reconstituted with all T cells except Treg (Aluvihare et al. 2004; Darrasse-Jèze et al. 2006). Both models have revealed that fetal growth is impaired exclusively in allogeneic matings secondary to the absence of Treg and that this is accompanied by an overwhelming infiltration by CD3$^+$ T cells at the maternal-fetal interface (Aluvihare et al. 2004). Other murine models confirm the importance of Treg cells in pregnancy maintenance. Adoptive transfer of Treg cells obtained from mothers pregnant after a non-abortion-prone mating (BALB/C $\times$ CBA/J) prevents fetal resorption in CBA/J (H-2k) female mothers pregnant after abortion-prone mating with DBA/2J (H-2d) males (Zenclussen et al. 2005). In humans, the number and proportion of Treg cells in maternal peripheral blood increases during early pregnancy, reaches its peak in the second trimester, and declines to its original level during the postpartum period (Somerset et al. 2004). Treg cells are highly concentrated in the decidua during early pregnancy, and lower numbers of peripheral and decidual Treg cells are reported among women suffering recurrent pregnancy loss when compared to controls (Sasaki et al. 2004). In pregnant mice, the expansion of Treg cells in systemic lymphoid organs begins prior to implantation, and these changes occur after both allogeneic and syngeneic matings (Aluvihare et al. 2004). This suggests that the increase in pregnancy-induced Treg cells is not a consequence of exposure to paternal antigen but a more nonspecific alteration of maternal immunity, possibly a hormonal influence.

8 Putative Molecular Mechanisms Involved in Immune Tolerance During Pregnancy

As our understanding of the molecular mechanisms involved in peripheral innate and adaptive immunity and the development of tolerance continues to grow, these and related pathways are being explored at the maternal-fetal interface. Of particular interest for their role in maternal-fetal tolerance are those mechanisms that have been linked to the development of immune suppression, including

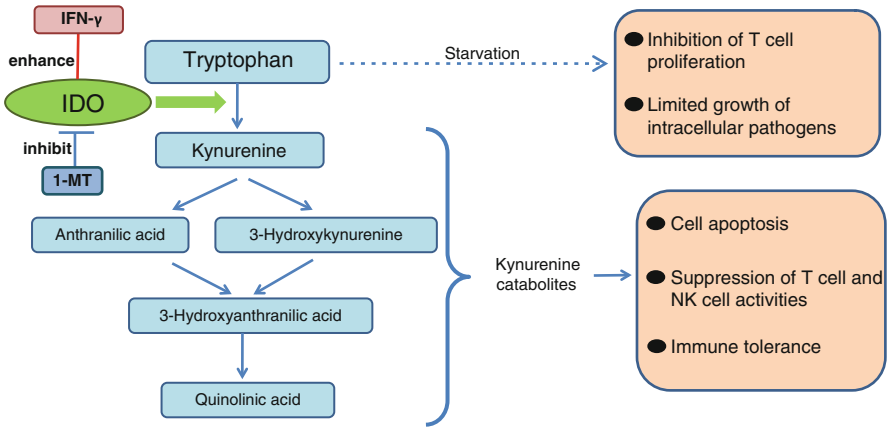

Fig. 4 The biologic consequences of tryptophan metabolism**.** Tryptophan can be metabolized by indoleamine-2,3-dioxygenase (IDO) to form kynurenine. Decreases in tryptophan inhibit T cell proliferation and limit growth of specific pathogens. Kynurenine and its catabolites can induce apoptosis and promote immune suppression and tolerance. IDO activity is enhanced by interferon gamma (IFN-γ) and inhibited by 1-methyl tryptophan (1-MT)

tryptophan/indoleamine-2,3-dioxygenase (IDO) pathways, B7-H1 / programmed death 1 (PD-1) interactions, Fas-Fas ligand (FasL) interactions, and the glycan-binding protein, galectin.

IDO catalyzes the initial and rate-limiting step in a tryptophan degradation process called the kynurenine pathway (Fig. 4). Increases in IDO activity result in local depletion of tryptophan and its downstream metabolites. The final biological effects of IDO depend upon this local depletion of tryptophan and its catabolites; effective depletion is a potent suppressor of T cell and microbe proliferation. Pharmacologic inhibition of IDO activity in mice enhances T cell activation and induces rapid rejection of allogeneic conceptuses (Munn et al. 1998). This can be reversed upon refeeding with tryptophan. Most investigators agree that IDO is expressed at the maternal-fetal interface. During pregnancy, IDO has been found in the maternal decidua (macrophages and stromal cells) and in the discrete population of invasive trophoblast cells that come into direct contact with maternal immune cells (giant cell trophoblast in mouse and EVTs in human placenta) (Baban et al. 2004; Hönig et al. 2004; Heikkinen et al. 2003; Kudo et al. 2004). Pointing again to the remarkable redundancy in fetal protective mechanisms, mice congenitally deficient in IDO show normal reproductive outcomes after allogeneic mating (Baban et al. 2004).

B7-H1 is a member of a large family of inhibitory, co-stimulatory cell surface molecules; its specific ligand on activated T cells is programmed death 1 (PD-1). During antigen recognition between APC and T cells, secondary engagement of PD-1 by B7-H1 inhibits primary signaling via MHC-TCR interactions, thereby suppressing T cell activation (Freeman et al. 2000). The importance of PD-1/B7-H1 interactions in maintaining peripheral tolerance is highlighted in the PD-1-deficient

mouse, which develops a lupus-like autoimmune disease and autoimmune dilated cardiomyopathy (Nishimura et al. 1999; Nishimura et al. 2001). The relevance of PD-1/B7-H1 signaling to T cell suppression during pregnancy is also seen using murine models. Blockade of B7-H1 signaling during murine pregnancy increases the rejection rates of allogeneic, but not syngeneic, conceptuses (Guleria et al. 2005). In humans, EVTs and decidual stromal cells constitutively express B7-H1, giving them the ability to suppress maternal T cell cytokine production through PD-1 signaling (Nagamatsu et al. 2009; Taglauer et al. 2008) and suggesting a significant role for this pathway in maintaining maternal-fetal tolerance in humans as well as mice.

Fas ligand (FasL) is a type II membrane protein and a member of the tumor necrosis factor superfamily. Its binding to Fas induces an apoptotic effect on Fas-bearing cells (Nagata and Golstein 1995). The spontaneous murine mutants, *lpr* and *gld,* are known to have loss of function mutations for Fas and FasL, respectively (Nagata and Suda 1995). Both models develop generalized lymphoproliferative diseases. Since FasL is mainly expressed on NK cells and activated T cells, findings in these mice suggest that the Fas-FasL system plays an essential role in the regulation of lymphocyte activation. Pregnant mice and pregnant women express FasL at the maternal-fetal interface on trophoblast and decidual cells (Hunt et al. 1997; Uckan et al. 1997). A series of studies using a murine model with transgenic expression of an H-Y-specific TCR have helped to elucidate the role of the Fas/FasL system in T cell tolerance to fetal antigen. When mice transgenic for the H-Y specific TCR became pregnant and the H-Y antigen was expressed only by male fetuses, a 50% reduction of H-Y-specific maternal T cells was noted. Further, the remaining T cells were hyporesponsive to H-Y antigen with reduced proliferative and cytotoxic capacities. These effects were abrogated in TCR transgenic females that were deficient in Fas (*lpr*) or FasL (*gld*) (Jiang and Vacchio 1998; Vacchio and Hodes 2003, Vacchio and Hodes 2005). Using embryo transfer experiments, Vacchio and Hodes (Vacchio and Hodes 2005) demonstrated that FasL expression by the fetus, but not by the mother, is critical for T cell deletion and hyporesponsiveness in murine models. The relevance of corticotrophin-releasing hormone (CRH) in FasL expression at the maternal-fetal interface has also been studied. Female rats treated with a CRH receptor type 1 antagonist during allogeneic matings exhibited a reduction in implantation sites and embryo viability; this effect was not observed after syngeneic matings or in T cell-deficient females. It was therefore proposed that CRH indirectly suppresses antigen-specific T cell responsiveness at the maternal-fetal interface by enhancing FasL expression (Makrigiannakis et al. 2001). As with other data addressing pregnancy-induced immune tolerance, the fact that both *lpr* and *gld* mice can propagate indicates that FasL/Fas interactions may be important, but are certainly not indispensable for the survival of allogeneic fetuses.

Galectin-1 (Gal-1) is a secreted glycan-binding protein with immunosuppressive properties. Gal-1 induces apoptosis of activated $CD8^+$ T cells (Perillo et al. 1995) and supports anti-inflammatory responses by promoting a Th2-type cytokine shift (Toscano et al. 2006). Gal-1-deficient pregnant mice exhibit elevated rates of fetal resorption after allogeneic matings but not syngeneic matings (Blois et al. 2007).

Interestingly, a microarray gene expression study found that decidual NK cells produce much higher levels of Gal-1 when compared with matched peripheral NK cells (Kopcow et al. 2008). The same study revealed that decidual T cells, but not peripheral T cells, possess a Gal-1-sensitive glycophenotype (Kopcow et al. 2008). Taken together these studies suggest that dNK cells may promote local depletion of alloreactive T cells through the secretion of Gal-1.

9 Antigen-Presenting Cells in the Decidua

Approximately 10% of the total number of human decidual cells and 20% of the decidual leukocytes are professional antigen-presenting cells (APCs; macrophages and dendritic cells) (Bulmer and Johnson 1984; Lessin et al. 1988). These cells are recruited from the periphery as circulating maternal monocytes and mature in situ into tissue-specific APCs under the influence of the local decidual microenvironment. The majority of decidual APCs exhibit an immature phenotype with relatively weak expression of activating co-stimulatory molecules and MHC class II (Heikkinen et al. 2003; Repnik et al. 2008). More than 90% of MHC class II-positive cells in the human decidua are also $CD14^+$ (decidual macrophages); $CD14^-HLA^-DR^+$ dendritic cells comprise less than 2% of the MHC class II-positive decidual cells (Gardner and Moffett 2003). In contrast to their peripheral counterparts, decidual macrophages exhibit immunosuppressive properties, characterized by abundant production of IL-10, an anti-inflammatory cytokine, and efficient IDO activity (Heikkinen et al. 2003; McIntire et al. 2008). Mixed lymphocyte reactions conducted in the presence of decidual macrophages show a marked inhibition of T cell activation (Ishihara et al. 1991; Parhar et al. 1989). The combination of enhanced immunosuppressive activities and a diminished capacity for antigen presentation by decidual APCs is thought to be advantageous for the establishment of local immune tolerance in the maternal uterus. These specialized cells may exert other activities that are beneficial to pregnancy but not directly associated with antigen presentation. Investigators have recently shown that conditional ablation of DCs in the murine decidua resulted in a striking impairment in implantation after syngeneic and allogeneic matings. The same was true after similar ablations in T cell-deficient animals. Implantation failure in these animals was attributed to a derangement in decidual vascular development (Plaks et al. 2008). Decidual APCs may have a role in the establishment of utero-placental circulation that equals or surpasses their expected role in regulating maternal T cell responses to fetal antigens.

10 Hormonal Regulation

Sex steroid hormones are potent modulators of the immune and endocrine environment during pregnancy. Estrogen and progesterone are the dominant sex steroids in the pregnant female. They begin their rapid rise after ovulation and, if conception

occurs, continue this rise throughout gestation, falling only after delivery of the placenta. These dramatic hormonal changes are thought to be important in creating an immunological and endocrinological milieu favorable for pregnancy maintenance. They are certainly involved in the increased susceptibility of the pregnant mother to specific pathogen insults (see below). Through interactions with classical cytosolic and nuclear receptors (and more recently described membrane-associated receptors), estrogen and progesterone control immunity directly by inducing changes in cell proliferation and activation level and indirectly by modulating the production of cytokines and growth factors in immune cells.

Naïve T helper cells (Th0) are induced to differentiate along a variety of pathways by the cytokine and endocrinologic microenvironment present when they meet their cognate antigen. These pathways have been generally subdivided into Th1, Th2, Th17, and Treg pathways, each characterized by the secretion of a specific subset of cytokines. It is well accepted that pregnancy is characterized by a shift toward both peripheral and local production of IL-4, IL-5, and IL-10, better known as a T helper cell type 2 (Th2) bias (Lin et al. 1993). Th2 cytokines are generally pro-tolerogenic, and Th2 cells inhibit the production of inflammatory Th1 cytokines. The importance of the Th2 bias in pregnancy is supported by the detrimental effects seen after Th1 cytokine administration during murine pregnancy (Raghupathy 1997).

Progesterone promotes the production of the transcription factor driving Th2 differentiation and thereby the production of Th2-type cytokines (Piccinni et al. 1995). Progesterone also drives the secretion of leukemia inhibitory factor (LIF), one of the few chemokines shown to be absolutely essential for embryo implantation (Piccinni et al. 1998). There is a growing series of papers that describe a somewhat enigmatic substance, called progesterone-induced blocking factor (PIBF) that acts as a secondary mediator of progesterone during pregnancy. PIBF promotes Th2-type cytokine production by binding to a novel IL-4 receptor and dampens NK cell-mediated cytotoxicity through the inhibition of perforin degranulation (Faust et al. 1999; Kozma et al. 2006). While this factor has been studied for many years, its molecular identity remains elusive. Many of the autoimmune effects previously attributed to Th1 cells are now being more specifically linked to Th17 cells (Nakae et al. 2007). Th17 cells are characterized by the production of IL-17 and are central to neutrophil recruitment and activation (Vanden Eijnden et al. 2005). Th17 cells comprise a higher proportion of decidual lymphocytes in normal pregnant women when compared to matched peripheral blood samples (Nakashima et al. 2010), and peripheral and decidual Th17 cell numbers are higher among women with unexplained recurrent pregnancy loss when compared to normal early-pregnant and to nonpregnant controls (Liu et al. 2011). There is indirect evidence that estrogen exposure induces Th17 differentiation of naïve $CD4^+$ cells (Wang et al. 2010). Interestingly, Th17 cytokines promote epithelial cell production of antimicrobial peptides (Aujla et al. 2007; Kollmann et al. 2009), a process that may deter unfettered immune activation in the amniotic cavity when breached by limited numbers of certain microbes (Witkin et al. 2011).

While the effects of progesterone on promoting Th2 differentiation and an anti-inflammatory environment in the pregnant mother are well accepted, the immune effects of estrogens during pregnancy are considerably more complicated and pleiotropic. Estrogens are generally thought to promote autoimmunity and have been considered the primary factor involved in the dramatic female/male bias in the incidence of several autoimmune disorders, including Th2-mediated diseases like the systemic lupus erythematosus (González et al. 2010). Those estrogen-related immune changes that would be predicted to promote pregnancy maintenance include the inhibition of delayed-type hypersensitivity and the promotion of Th2-type immune responses (Correale et al. 1998; Cutolo and Wilder 2000; Gregory et al. 2000; Knoferl et al. 2000; Müller et al. 1999; Salem et al. 2000). The latter has been shown to protect against chronic allograft rejection (Müller et al. 1999), but will predictably worsen Th2-mediated autoimmune diseases during pregnancy. As for the pleiotropic effects of estrogen on immune responsiveness, the IFN-γ promoter contains an estrogen response element, and enhanced production of this Th1 cytokine (i.e., IFN-γ) occurs following estrogen treatment in lymphoid cells (Fox et al. 1991). Estrogen appears to have somewhat unusual promoting effects for both Th1- and Th2-type responses. Using mouse models, investigators have recently demonstrated that Treg expansion during pregnancy is induced by estrogen and progesterone. The presence of estradiol and progesterone stimulated the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells and increased Foxp3 and IL-10 expression in ovariectomized mice (Mao et al. 2010; Tai et al. 2008).

The activation level and functional differentiation of T cells are determined by the characteristics of their interactions with APCs (types of primary receptors and co-receptors) as well as the constituents in the local cytokine milieu. Several studies have reported that T cells express cytosolic and nuclear estrogen receptors and membrane progesterone receptors. Most unstimulated T cells do not appear to express classical nuclear progesterone receptors (Schust et al. 1996). However, it may be that the functional changes in T cells that are noted during pregnancy are not completely explained by direct hormonal effects. Rather, they may rely heavily on their interactions with specialized decidual APCs within a pregnancy-specific cytokine microenvironment.

In contrast, myelomonocytic cells, including macrophages and DCs, do express classical and membrane-bound estrogen and progesterone receptors and respond directly to hormonal stimulation (Nalbandian and Kovats 2005). Treatment with estradiol diminishes LPS-induced NF-κB expression in macrophages, leading to reduced production of the proinflammatory cytokines IL-1α and IL-6 (Deshpande et al. 1997). Estradiol treatment also enhances DC production of CC chemokine ligand 1 (CCL1), which can attract CCR8-expressing Th2 cells and regulatory T cells (Uemura et al. 2008). Progesterone but not estrogen acts as a potent inhibitor of macrophage-derived TNF-α, a substance that may otherwise have detrimental effects on fetal growth. Progesterone exerts these effects by limiting NF-κB function through the upregulation of I-κB expression, thereby causing a reduction in TNF-α transcription (Miller and Hunt 1998). Treatment of DCs with progesterone reduces cell surface expression of MHC class II and the co-stimulatory

molecule CD80 as well as the secretion of the proinflammatory cytokines IL-1β and TNF-α. In addition, progesterone inhibits DC-stimulated proliferation of T cells, suggesting that progesterone reduces the capacity of DCs to drive downstream inflammatory processes (Butts et al. 2007). Although not proven, the combined effects of gestational steroid hormones on myelomonocytic cells may promote functional differentiation of decidual APCs into immunosuppressive and tolerogenic phenotypes during pregnancy.

11 Susceptibility to Infectious Pathogens During Pregnancy

Drastic changes in maternal immune function during pregnancy typically combine with trophoblast tolerogenic mechanisms to create a redundant but harmonized maternal response to fetal antigen. These same immunologic changes alter the protective mechanisms exquisitely refined to prevent infectious diseases under nonpregnant conditions. Somewhat surprisingly, the intrauterine environment is not sterile (Witkin et al. 2011). Therefore, in addition to balancing allograft protection against pathogen invasion, immune cells at the maternal-fetal interface must control the growth of potential pathogens while avoiding an overwhelming response to these invaders that might escalate into premature rupture of membrane and preterm labor and delivery.

The quality and quantity of systemic and local alterations in maternal immunity depend on gestational age-specific factors, including the local and circulating levels of gestational hormones and other placenta-derived substances as well as the anatomical structure of the utero-placental unit. Depending on the point in pregnancy at which infection occurs, invasion by a given pathogen may result in quite varied maternal clinical manifestations and maternal-fetal transmission rates. Several infectious organisms appear to have mechanisms that allow them to capitalize on the immunologic vulnerabilities of the pregnant female. In the following sections, we will discuss the unique course of several infectious pathogens during pregnancy, including bacterial causes of preterm birth, cytomegalovirus and parasitic infections, toxoplasmosis, leishmaniasis, and malarial infections. We will focus on pathogen-specific molecular strategies for immune evasion within the microenvironment of the pregnant uterus and on the unique characteristics of the provoked immune responses at the maternal-fetal interface (Table 1).

11.1 Bacterial Infection as a Cause of Preterm Birth

Preterm birth (PTB) accounts for 12–13% of all births in the United States but results in 70% of perinatal mortality and 50% of long-term morbidity (Martin et al. 2010; McCormick 1985). Although the survival rate for neonates born prematurely has improved secondary to advances in their pediatric management, there has been little or no decline in the rate of preterm delivery over the last several decades

Table 1 Major infectious diseases during pregnancy

	Major pathogen	Route of infection	Clinical features and consequences	Prenatal treatment choice
Chorioamnionitis	Variety of vaginal flora bacteria, periodontal bacterium	Trans cervical invasion, hematogenous spread, iatorogenic	Preterm birth following preterm labor and rupture of membranes, sepsis in the neonate	Antibiotics
Syphilis	*Treponema pallidum*	Sexual transmission	Maternal cutaneous symptoms, large edematous placenta, congenital anomalies, fetal loss, fetal growth restriction	Penicillin G
Cytomegaloviral Infection	Human cytomegalovirus	Contact with infectious body fluid, reactivation of virus	No apparent maternal symptoms, fetal ventriculomegaly, microcephaly, hepatosplenomegaly, growth restriction	Treatment strategy is not established. Prenatal administration of intravenous hyperimmune globulin and anti-viral agents is reported
Toxoplasmosis	*Toxoplasma gondii*	Oral intake of tissue cysts, Contact with cat feces containing oocysts	Asymptomatic in most of mothers. Neonatal intracranial and retinal lesions associated with neurological impairment and retinochoroiditis	Spiramycin
Leishmaniasis	*Leishmania donovani*, *L. chagasi*, rarely *L. tropica*, *L. mexicana*	Bite of female sandfly	Kala-azar (darkening of the skin), splenomegaly, hemorrhage, anemia in the mother. Vertical transmission is possible	Antimony, Amphotericin B (Safety for pregnant women is not determined.)
Malaria	*Plasmodium malariae, P. ovale, P. vivax,* and *P. falciparum*	Bite of female Anopheles mosquitos	Maternal anemia, placental malaria, fetal growth restriction, miscarriage, fetal loss	Anti-malarial drugs

(Martin et al. 2010). There are many potential triggers for PTB; intrauterine bacterial infections represent the leading causative factor (Lettieri et al. 1993). PTBs associated with bacterial infection are also more likely to occur at earlier gestational ages, making the risk for maternal and perinatal morbidity and mortality even more immediate (Goldenberg et al. 2000).

The primary route of access for bacteria causing PTB is egress from the vagina and through the uterine cervix. This is supported by accumulating evidence showing an increased risk for PTB among women with bacterial vaginosis (Donders et al. 2009; O'Brien 2005). Bacterial invasion can also occur after hematogenous spread from other sites of infection (e.g., periodontal infection) or, iatrogenically, after invasive medical procedures (e.g., amniocentesis or chorionic villus sampling). Culture analyses using amniotic fluid and chorioamniotic membranes obtained from cases of PTB have identified a variety of potentially causative bacterial species. The most common species isolated from women who had PTB after either spontaneous rupture of the membranes or in the presence of intact membranes are *Ureaplasma urealyticum*, *Mycoplasma homini*, peptostreptococci, and *Gardnerella vaginalis* (Pararas et al. 2006). All of these organisms are typically of low virulence and are present as minor components of normal vaginal flora under lactobacilli dominance. Among women with PTB after membrane rupture, *Neisseria gonorrhoeae*, group B streptococci, *Escherichia coli*, and *Chlamydia trachomatis* may be involved as causative pathogens (Pararas et al. 2006). The fact that a wide array of causative organisms culminate in an identical clinical consequence, preterm birth, suggests a shared inflammatory pathway leading to uterine contractions and membrane rupture that follows initial incitement of more varied mechanisms for pathogen-specific activation of local immune cells.

Bacterial endotoxins and exotoxins stimulate the production of proinflammatory cytokines from cells within the decidua and fetal membranes. Pregnant mice exposed to bacteria-derived ligands for toll-like receptors (TLRs) first exhibit enhanced local and uterine production of proinflammatory cytokines and subsequently develop preterm labor and preterm birth (Ilievski et al. 2007; Koga and Mor 2010). During human pregnancies, elevated concentrations of TNF-α, IL-1α, IL-1β, IL-6, and IL-8 in amniotic fluid have been reported as predictive markers for preterm labor. Among these cytokine markers, TNF-α and IL-1β potently stimulate the local production of prostaglandins (Estrada-Gutierrez et al. 2010; Sadowsky et al. 2006; Vadillo-Ortega et al. 2002). Prostaglandins, in turn, induce uterine contractions and the release of matrix-degrading enzymes that facilitate membrane rupture and remodeling of the uterine cervix (Estrada-Gutierrez et al. 2010; Sadowsky et al. 2006; Vadillo-Ortega et al. 2002). Notably, mice lacking the receptors for TNF-α and IL-1β (TNF-αR and IL-1βR double knockouts) do not experience preterm labor upon LPS administration, confirming a critical role for these particular cytokines in the pathological process of PTB (Hirsch et al. 2006). Also of interest, IL-6 may actually be a marker of the intrauterine protective response to the presence of bacterial invaders, possibly rising after bacterial invasion to help delimit overwhelming inflammation in the intrauterine environment that might otherwise cause preterm labor and birth (Witkin et al. 2011). IL-6 reduces neutrophil migration to sites of inflammation (Fielding et al. 2005), and

IL-6 instillation into the amniotic cavities of pregnant rhesus macaques does not cause preterm labor and delivery (Sadowsky et al. 2006).

As mentioned previously, the amniotic cavity and fetal membranes are not consistently aseptic during normal human pregnancy. In fact, using culture analyses and PCR, the presence of bacteria has been confirmed in amniotic fluid (AF) obtained from 10% to 20% of uncomplicated pregnancies; that rate was significantly higher among pregnancies complicated by PTB (Gerber et al. 2003; Horowitz et al. 1995; Onderdonk et al. 2008; Steel et al. 2005). It appears the presence of bacteria alone may not be sufficient to cause PTB. Specific fetally derived mechanisms to avoid unfettered immune response to invading bacteria have been recently reviewed by Witkin et al. (2011); we will concentrate mainly on maternal adaptations here. Still, the specific combination of the immune-related genetic backgrounds of the mother and the fetus may significantly influence the risk for PTB in any given pregnancy. Using single-nucleotide polymorphism (SNP) analysis to examine the association between the maternal and fetal cytokine genotypes and the concentration of these cytokines in the amniotic fluid of PTB cases, SNPs in the genes coding IL-1β, IL-6, TNF-α, and IL-10 were noted to partially account for their amniotic fluid concentrations (Menon et al. 2008, 2010; Velez et al. 2007). These studies also suggested that the contribution of SNPs to cytokine concentrations in PTB may help to explain ethnic differences in the rates of PTB among African Americans and Caucasians (Menon et al. 2008, 2010; Velez et al. 2007). Of course, the underlying maternal immunological status will also determine the risk of PTB, with increased frequency of PTB noted among immunocompromised pregnant women (e.g., women with diabetes mellitus and AIDS and women chronically using glucocorticoids)(Brocklehurst and French 1998; Laskin et al. 1997; Sibai et al. 2000).

Clinically, intrauterine infections are often fairly silent early in their course. They are also fairly common, as evidenced by the frequent presence of mild chorioamnionitis in normal term deliveries (Potkul et al. 1985). Standard inflammatory symptoms such as pain, fever, and swelling are typically not seen until just prior to the onset of uterine contractions, rupture of membranes, and unstoppable labor. However, studies using amniotic fluid obtained during the midtrimester have demonstrated that an increase in proinflammatory mediators can be fairly easily detected during the asymptomatic phase of PTB (Baud et al. 1999; Romero et al. 1990). This supports the concept that immune cells at the maternal-fetal interface respond to bacterial invasion at a subclinical level and raise hope that early diagnosis of intrauterine infection may be possible during this silent battle against intrauterine infection. Early intervention might then prevent the subsequent intense inflammatory response that triggers the onset of labor, thereby extending the gestational period and preventing or ameliorating fetal morbidity and mortality. There are several mechanisms that may obfuscate early symptoms of intrauterine pathogen invasion. Most are likely protective to allow low-level invasion, and both mother and fetus must certainly cooperate in this pregnancy protection. The maternal mechanisms likely overlap with those that aid in the acceptance of the fetal allograft, including changes in gestational hormones and alterations in tryptophan metabolism.

As previously discussed, investigations using competitive IDO inhibition revealed that IDO is involved in blocking T cell-mediated rejection of allogeneic fetuses (Munn et al. 1998). IDO inhibition deprives the T cells of the essential amino acid, tryptophan, which is necessary for T cell proliferation (Taylor and Feng 1991). Since tryptophan is also essential for the growth and development of several types of bacteria, pregnancy-related decreases in IDO activity will also inhibit pathogen survival within the reproductive tract. Reproductive pathogens affected by IDO inhibition and tryptophan depletion include intracellular bacteria such as *Chlamydia* and *Listeria* and extracellular bacteria such as streptococci, enterococci, and staphylococci (MacKenzie et al. 2007). In short, IDO inhibition appears to serve dual functions, controlling bacterial invasion of the maternal reproductive tract while promoting maternal T cell tolerance to the allogeneic fetus.

Progesterone displays multiple activities that support pregnancy maintenance. Progesterone prevents uterine contractions by modulating the local inflammatory response to invading microorganisms. Progesterone-treated DCs have a reduced ability to present antigens. Progesterone directly and/or indirectly suppresses the activation level of T cells and NK cells. In vitro investigations using the macrophages stimulated with LPS and CpG oligodeoxynucleotides (ligands for TLR4 and TLR9) have shown that progesterone restricts TLR-mediated signaling (Su et al. 2009). Progesterone-induced suppression of TLR signaling contributes to reductions in inducible NO synthesis and an inhibition of NF-κB secondary to enhanced expression of the suppressor of cytokine signaling (SOCS) (Su et al. 2009). This immunomodulatory effect of progesterone is mediated by both glucocorticoid receptors and progesterone receptors (Jones et al. 2008). All of these anti-inflammatory effects may aid in protection from excessive immune responses that could directly damage the fetal allograft or stimulate preterm labor and preterm fetal expulsion. The salutary effects of progesterone on pregnancy are further supported by accumulating clinical evidence that prophylactic administration of progesterone in patients at risk for PTB prolongs pregnancy if initiated between 16 and 24 weeks of gestation and begun prior to the onset of symptoms; initiation of treatment at an advanced stage of preterm labor does not extend the gestational period (Dodd et al. 2008). Whether the mechanisms are direct or indirect and regardless of use of classical, nonclassical, or cross-reactive receptors, progesterone desensitizes the systemic and local immune reactivity of the pregnant mother. This raises the local threshold for triggering antibacterial inflammatory responses that can lead to PTB, exquisitely balancing maternal antibacterial immunity with protection from preterm labor and with maternal tolerance of pregnancy.

11.2 Cytomegaloviral Infection

Cytomegalovirus (CMV) is a double-stranded DNA virus that belongs to the *Herpesviridae* family. While infection can be deadly in the immunocompromised, it causes subclinical infection and establishes a lifelong latency in the vast majority

of immunocompetent hosts. Women who have not been infected with CMV prior to pregnancy are at significant risk for transplacental passage should their first exposure occur while pregnant. CMV is among the most common of the congenital viral infections, with an overall incidence of 0.2–2% (Leung et al. 2003). The maternal-fetal transmission rate is reported to be 30–40% for primary infections and 1% after CMV reactivation from latency (Kenneson and Cannon 2007). The clinical impact of congenital CMV infection on the fetus is variable and depends on the time at which infection or reactivation occurs during pregnancy. In general, transmission rates appear to be greater when infections occur late in pregnancy, but the fetal effects are typically worse when infection occurs early in pregnancy. Some exposed neonates are asymptomatic at birth; fetal growth restriction and stillbirth are more common after exposures in the first and second trimesters. In clinical settings, CMV infection of the placenta is confirmed only after delivery, when viral inclusions are found on histopathological examination of the placenta and fetal membranes.

Extensive investigations on the molecular function of CMV gene products have shown that CMV has evolved multiple strategies to escape human immune surveillance. HLA products are central to host recognition of viral antigens and subsequent control of infection by cytotoxic $CD8^+$ T cells and cytolytic NK cells. Many of the strategies utilized by CMV for immune evasion are directed at HLA-mediated host-protective pathways. These immune evasion mechanisms involve a series of CMV-encoded molecules that are expressed at different stages of infection and categorized as unique short (US) and unique long (UL) proteins. For instance, the CMV-encoded molecules US2, US3, US6, US10, and US11 interfere with several aspects of the intricate process involved in assembling the mature MHC class I antigen-presenting complex for expression at the cell surface (Schust et al. 1998; Tortorella et al. 2000). Impairment in host production of the MHC class I heavy chain, peptide antigen loading of the heavy chain, and association of heavy chain with the invariant light chain, β2-microglobulin (β2m), in the endoplasmic reticulum lead to the diminished ability of the infected host cells to alert immune cells to pathogen invasion. This strategy enables CMV to escape recognition by $CD8^+$ cytotoxic T cells. However, since NK cells have an ability to recognize virally infected cells that display reduced HLA class I and increased stress-inducible molecules (Binstadt et al. 1997; Seliger et al. 2006), this would potentially increase susceptibility of the infected cell to NK cell-mediated cytolysis. To avoid this, the virus has cultivated additional strategies to elude NK cell-mediated antiviral defenses. CMV synthesizes a decoy molecule, called UL18, that shares 25% of its amino acid sequence with the extracellular region of HLA class I and thereby functions as an HLA class I homologue. UL18 does not present CMV-derived antigens to cytotoxic T cells but associates with β2m and binds to immunoglobulin-like transcripts (ILTs) as a substitute for its endogenous ligand, the HLA class I-β2m complex. The affinity of UL18 for the inhibitory receptor, ILT2, is higher than that of HLA class I, suggesting that low-level expression of UL18 can efficiently prevent NK-mediated lysis of infected host cells (Chapman et al. 1999). CMV also secretes a protein called UL16 that selectively masks ligands for the activating NK cell receptor NKG2D, including MHC class I polypeptide-related sequence B (MICB), ultralong binding protein 1

(ULBP1), and ULBP2 (Dunn et al. 2003; Rölle et al. 2003). This also inhibits NK cell activation and helps to avoid lysis of CMV-infected cells.

Considering the dramatic alterations in pregnancy outcomes that sometimes occur after CMV infection, it is enticing to speculate that CMV has pregnancy-specific immune evasion strategies as well. Alternatively, the pregnant female may have adapted herself to avoid certain aspects of CMV-mediated immune evasion. Compared with dominant expression of HLA-A and HLA-B on somatic cells, the unique and restricted pattern of HLA class I products on EVTs (no HLA-A and HLA-B only HLA-C, HLA-E, and HLA-G) could influence the susceptibility of the maternal-fetal unit to CMV infection. US11-mediated HLA degradation does not affect HLA-E and HLA-G, because they lack an essential residue in their cytoplasmic tails that mediates interaction with US11 (Barel et al. 2003a, 2006; Schust et al. 1998; Tortorella et al. 2000). HLA-C and HLA-E are not targeted by US2-mediated degradation, while membrane-bound HLA-G is sensitive to US2 (Barel et al. 2003b; Gewurz et al. 2001; Schust et al. 1998; Tortorella et al. 2000). More generalized investigations assessing the combined effects of CMV immune evasion gene products on HLA-G expression have demonstrated preservation of HLA-G, but not of HLA-A, at the cell surface upon infection with CMV (Pizzato et al. 2004; Terauchi et al. 2003). Preserved expression of HLA-G on EVTs during infection may help to extend the survival of CMV since the pregnancy maintenance-related immunosuppressive function of HLA-G could protect even infected host cells from cytotoxic immune cells. CMV also encodes a protein called UL40 that contains a leader sequence that is highly similar to that of HLA class I molecules. Since HLA-E loads HLA-derived class I peptides in its peptide-binding groove as a means to monitor class I molecule production in the cell, the presence of UL40-loaded HLA-E could function as a surrogate cell surface signal when endogenous leader peptide availability is restricted by the action of CMV products such as US6 that degrade trophoblast-expressed MHC class I molecules (Tomasec et al. 2000). Indeed, UL40 appears to help CMV-infected cells avoid lysis by NK cells by allowing HLA-E ligation with the CD94/NKG2A inhibitory receptor on NK cells despite reduced HLA class I production (Ulbrecht et al. 2000).

In short, immune changes at the human maternal-fetal interface may ameliorate some immune evasion strategies of CMV, but worsen others. CMV may have targeted some of these vulnerabilities during coevolution with its host, resulting in a pregnancy-specific vulnerability to infection and transmission that makes CMV the most common congenital viral pathogen.

11.3 Parasitic Infections

Similar to the gender-biased susceptibility to autoimmune diseases, gender-biased differences in the susceptibility to and severity of several parasitic diseases are thought to rely heavily on differences in circulating sex steroids. This sex-associated dimorphism may be exaggerated during the gestational period when sex-steroid-mediated maternal immune responses shift toward immune suppression and allograft tolerance. The Th2-biased cytokine milieu, the suppressed cytolytic

ability of decidual NK cells, and the tolerogenic properties of decidual macrophages at the maternal-fetal interface combine to dampen the efficiency of maternal antiparasitic immune responses, thereby increasing the risk of persistent infection and the likelihood of pathogen transmission to the fetus.

11.3.1 Toxoplasmosis

Toxoplasma gondii (*T. gondii*) can cross the placenta and cause congenital toxoplasmosis when maternal infection occurs during pregnancy. Maternal parasitemia most commonly occurs following the intake of tissue cysts residing in improperly cooked meat from infected animals or upon the contact with cat feces containing oocysts. The risk for maternal-fetal transmission and the severity of subsequent fetal damage are closely associated with the gestational age at the time of maternal parasitemia. Like infections with CMV, vertical transmission is not frequent in the early pregnancy, but the impact on the infected fetus can be quite severe. Transmission rates increase with increasing gestational age at the time of maternal parasitemia, reaching 60–80% at term. Late infections are associated with mild fetal effects. Histologically, placental infection is characterized by intense granulomatous villitis and the presence of toxoplasma trophozoites.

Pregnancy increases maternal susceptibility to toxoplasma infection. In murine models, the lethal dose of tachyzoites is significantly lower in pregnant mice when compared to virginal mice (Shirahata et al. 1992). Infection-induced production of IFN-γ, a major host mediator of toxoplasma control, is depressed in pregnant mice, while the administration of Th1-type cytokines (IFN-γ and IL-2) decreases parasitic growth in pregnant mice and reduces maternal mortality (Shirahata et al. 1992, 1993). Pregnancy-associated cytokine changes that include suppression of IFN-γ production and a Th2 bias will predictably compromise the mother's ability to respond to and overcome intracellular *T. gondii* parasites.

DCs and macrophages are also involved in the initial recognition of toxoplasma invasion. Toxoplasma tachyzoites express several cell surface molecules that are recognized by immune cells early after infection. Binding of host TLR11 with a tachyzoite-expressed profilin-like molecule and binding of host TLR2/TLR4 with glycosylphosphoinositol-anchored proteins on the tachyzoite surface trigger the production of IL-12 and TNF-α by DCs and macrophages (Debierre-Grockiego et al. 2007; Yarovinsky et al. 2005). Local increases in these proinflammatory cytokines couple with abundant IFN-γ secretion to create a Th1-dominant cytokine milieu and promote potent NK cell-mediated cytotoxicity. The proinflammatory effects of advanced infection may lead to pregnancy wastage and preterm delivery.

Increased macrophage production of nitric oxide (NO) by inducible NO synthase (iNOS) combines with host cell IDO induction and tryptophan deprivation to combat many intracellular pathogens, including *T. gondii* (Scharton-Kersten et al. 1997; Silva et al. 2002). Elevations in IFN-γ and TNF-α further increase the expression level and functional efficiency of IDO and iNOS (Fujigaki et al. 2002; Yap and Sher 1999). The pregnancy-specific characteristics of decidual macrophages, including ample secretion of IFN-γ-inhibiting IL-10, a Th2 cytokine

bias (called M2 polarity for macrophages), and diminished NO synthesis, would be predicted to counteract several of the mechanisms central to fighting *T. gondii* infection. In short, the immune changes central to allograft tolerance during pregnancy greatly increase maternal vulnerability to toxoplasma.

11.3.2 Leishmaniasis

Leishmaniasis can present as isolated cutaneous or as systemic/visceral disease. Both are caused by a parasite that infects and replicates within macrophages. Leishmanial organisms are transmitted through the bite of the sand fly. Visceral disease in humans is primarily associated with infection by *Leishmania donovani* or *Leishmania chagasi* (a.k.a. *L. infantum*), but can rarely complicate infection by *L. mexicana* or *L. tropica*. Like *Toxoplasma gondii,* host defense against leishmania depends heavily on Th1-type responses. In fact, the majority of host cell strategies to surmount parasite survival are shared between *T. gondii* and *Leishmania*. One murine model for leishmaniasis uses a zoonotic leishmania strain, *Leishmania major,* that causes cutaneous disease. Pregnant mice infected with *L. major* develop larger cutaneous lesions with extended resolution periods when compared with nonpregnant infected mice. Stimulation of these mice with leishmania antigen reveals significant reductions in the production of IFN-γ and increases in the production of IL-4, IL-5, and IL-10 by spleen and lymph node cells from pregnant mice, suggesting that skewing toward a Th2-type response during pregnancy may be a cause for impaired resistance to *Leishmania major* (Krishnan et al. 1996). In stark contrast, pregnant hamsters infected with *Leishmania panamensis* are more resistant to the infection than their nonpregnant counterparts (Osorio et al. 2008). While this apparent discrepancy could be pathogen/model specific, there are other theories. Elevated estrogen levels during pregnancy enhance NO production in macrophages and neutrophils through increases in iNOS quantity and activity (Dai et al. 2009; Osorio et al. 2008). By itself, this would be predicted to reinforce defense against leishmania during pregnancy. In humans, epidemiological data on leishmaniasis do not indicate increased risk or exacerbation during pregnancy, although congenital transmission of *L. chagasi* has been reported in endemic areas (Figueiró-Filho et al. 2004; Meinecke et al. 1999). It is plausible that an estrogen-derived strengthening of maternal resistance against leishmania helps to compensate for the weakened Th1-biased cellular immunity seen during human pregnancy.

11.3.3 Malaria

Malaria is caused by plasmodium species and is transmitted to humans by the bite of female *Anopheles* mosquitoes. The parasite has a complex life cycle in both the mosquito and the human. Soon after infection, plasmodium sporozoites infect host hepatocytes, where they replicate asexually and differentiate into merozoites during

a relatively asymptomatic stage of the disease. Merozoites envelop themselves in a membrane derived from the cell membrane of the host hepatocyte to exit the liver (Sturm et al. 2006) and travel relatively undetected in the host bloodstream, infecting red blood cells and beginning the more symptomatic erythrocytic stage of the disease (Casares and Richie 2009). The most common human plasmodium species are *P. malariae*, *P. ovale*, *P. vivax*, and *P. falciparum*.

Although maternal malaria is largely asymptomatic, especially in high transmission areas (Nosten et al. 1991), pregnancies affected by the disease can be compromised by fetal growth restriction, preterm delivery, severe maternal anemia, and preeclampsia (Newman et al. 2003). Among the four most common types of human malarial parasites, *Plasmodium falciparum* poses the most serious threat to human health. It is well accepted that pregnant women are particularly vulnerable to infection by *P. falciparum* (Uneke 2007). Multiple factors have been suggested to explain this increased susceptibility. Pregnant women are more likely to be bitten by vector mosquitoes than nonpregnant women (Lindsay et al. 2000). This interesting finding is possibly secondary to increased maternal body surface area but is more likely to be secondary to increased CO_2 exhalation among pregnant females. Mosquitoes are attracted to CO_2. Once bitten, it is hypothesized that local and systemic pregnancy-related immunomodulation favors parasite expansion within the pregnant host. Primigravid status and pregnancy at a young age are independent risk factors for adverse perinatal outcomes among women suffering maternal malaria (Rogerson et al. 2000; Steketee et al. 1996). This tendency is especially evident in areas with high infection rates because repeated prior exposures to the parasite under pregnant or nonpregnant states provide multigravid and older women with additional opportunities to develop protective immunologic memory, including antibodies specific to adhesion molecules present on infected erythrocytes (IEs) (Fievet et al. 2002).

Epidemiological data from African populations has suggested that erythrocytic malarial parasites may undermine detection by host Treg cells (Torcia et al. 2008). The Fulani and Mossi tribes of Burkina Faso live in very similar geographic areas and have identical exposures to *P. falciparum*. Both tribes utilize similar antimalarial prevention strategies. The Fulani, however, are much more resistant to infection and malarial disease than the Mossi. Neither tribe has significant differences in the expression of malaria resistance genes. However, when compared to the Mossi, the Fulani have fewer and less active circulating Treg cells. Expression of TGFβ, TGFβ receptors, and FoxP3 is lower among the Fulani, as were circulating levels of TGFβ. Finally, the Fulani's proliferative response to malaria-derived antigens has been shown to be unchanged after Treg depletion, while that of the Mossi increased significantly. The known estrogen-driven increase in local and systemic Treg number and activity among pregnant women may make them particularly vulnerable to *P. falciparum* infection.

The placenta appears to provide a particularly important site for *P. falciparum* sequestration, allowing the parasite to proliferate freely while evading maternal antimalarial immunity (Matteelli et al. 1997). Since the majority of the parasites are sequestrated in the placenta, maternal parasitemia may not be detectable in the

blood of the pregnant host (Mockenhaupt et al. 2006). Pathological features of placental malaria include the accumulation of infected erythrocytes in the intervillous space, scattered areas of placental necrosis accompanied by infiltration of inflammatory cells, and the deposition of malarial pigment (Muehlenbachs et al. 2010). These histological findings reflect an impairment in placental function that ultimately leads to adverse outcomes, including fetal growth restriction and preterm delivery. Chondroitin sulfate A (CSA) appears to be key to the occurrence of parasite sequestration in the placenta. While most nonplacental isolates of *P. falciparum* bind to the scavenger receptor, CD36, on erythrocytes, they rarely bind to CSA. In contrast, placental isolates preferentially bind to CSA but not to CD36 (Fried and Duffy 1996). CSA is expressed on syncytiotrophoblast and functions as an adhesion molecule to facilitate the attachment of infected erythrocytes to the villous surface of the placenta (Fried and Duffy 1996). These findings indicate that a special subpopulation of *P. falciparum* isolates with novel variant surface antigens that bind to CSA selectively propagate in the human placenta. In fact, women who have already acquired natural immunity to *P. falciparum* can suffer lethal infections by placental malarial strains (Casares and Richie 2009). While antigenic variation may be an important immune evasion mechanism for *P. falciparum*, death of the host must certainly represent a suboptimal outcome. CSA-binding infected erythrocytes are phagocytosed by monocytes/macrophages at a lower level than nonplacental, CD36-binding infected erythrocytes (McGilvray et al. 2000; Serghides et al. 2006). Therefore, the atypical binding properties of placental infected erythrocytes may also interfere with innate immune mechanisms that clear parasites. These factors would be predicted to be additive or multiplicative in their effects on parasite accumulation in the placenta.

Inflammatory, Th1-type immunity toward *P. falciparum* does develop among infected pregnant women. Elevated levels of proinflammatory cytokines can be seen in placental serum, and increased expression of corresponding mRNAs in placental tissues has been reported among infected pregnant women (Fried et al. 1998; Moormann et al. 1999). Most investigators agree that there is an enhanced production of TNF-α in pregnant women infected with plasmodium species, particularly among those women who are also experiencing fetal growth retardation. Monocytes obtained from the placental blood of *P. falciparum*-infected women demonstrate an enhanced ability to produce IL-12 (Diouf et al. 2007). Monocyte-derived IL-12 induces Th1-type cytokine secretion, activating NK cells and prompting local production of IFN-γ (Ockenhouse et al. 1984). IFN-γ, in turn, facilitates macrophage-mediated killing of intraerythrocytic parasites (Ockenhouse et al. 1984). Pregnant women who display elevated IFN-γ secretion from intervillous mononuclear cells in response to malarial antigen are less likely to become malaria infected when compared with those who have lesser antigenic responses (Moore et al. 1999; Othoro et al. 2008). Should these women become infected, however, it might be predicted that they would be more likely to suffer adverse pregnancy outcomes. Overall, the gestational hormone-induced suppression of cellular immunity and the Th2-biased cytokine milieu of pregnancy should temper the Th1-type protective immune responses against malarial attack. To this point, the peak prevalence of malarial infection among pregnant women occurs,

regardless of parity, during the second trimester when local and systemic Th2 dominancy is fully established (Brabin 1983).

12 Conclusions

The mammalian placenta is a remarkable organ. It is confronted by a unique set of immunologic challenges: maintenance of tolerance to fetal antigens and simultaneous protection from pathogen invasion. The placenta orchestrates the changes in local and systemic immunity that must remain in delicate balance throughout pregnancy. While typically remarkably successful, this balance can occasionally be disrupted by maternal infection. Such infection may arise from local ascending spread through the female reproductive tract or from systemic insults. Parasitic infections and certain viral infections can be particularly aggressive during pregnancy.

The pregnant uterus is considered an immune-privileged site. Cooperation between fetally derived trophoblast cells and maternal decidual cells help to maintain this unique immune environment. Among the many strategies employed are alterations in local and systemic steroid hormone concentrations, altered trophoblast expression of molecules involved in antigen presentation, and population of the maternal decidua with a morphologically and functionally unique set of immune cells, including regulatory T cells, NK cells, macrophages, and dendritic cells. While protecting the fetus from harmful maternal alloimmunity, immune privilege makes the fetus vulnerable to pathogen attack. We have reviewed here the role of immune privilege at the maternal-fetal interface in bacterial infections causing preterm birth, in human cytomegaloviral infection, and in a variety of parasitic infections that can result in adverse pregnancy outcomes. While other pathogens, including herpes simplex virus, varicella virus, and *Treponema pallidum*, can be particularly aggressive when infection occurs during pregnancy, the chosen pathogens illustrate the immunologic challenges of pro-tolerogenic pregnancy-specific immune changes when infection arises. They demonstrate the need for tight regulation of the immune response to ascending pathogens from the reproductive tract in an effort to control pathogen entry without inducing a cascading maternal immune response that leads to preterm uterine contractions and preterm birth. Finally, the chosen pathogens illustrate the expansive strategies that pathogens use to evade host immune detection, some of which appear to have specifically evolved to aid in infection of the pregnant host.

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The Liver as an Immune-Privileged Site

Dirk Wohlleber and Percy A. Knolle

Abstract The liver is well known as an organ with important functions in the metabolism of lipids, proteins, and carbohydrates. Besides these functions, it is getting increasingly clear that the liver has also a central role in initiating and modulating immune responses. Special attention is linked to the fact that 75% of the liver's blood supply comes as venous blood from the gut, not only rich in nutrients but also rich in microbial degradation products. To protect the organ and the body from these immunostimulatory microbial products, the liver has an extraordinary scavenger function clearing all these molecules. At the same time, the liver itself is protected by establishing a tolerogenic state by modulating immune responses. Adaptive and innate immune reactions are suppressed to avoid unnecessary immune activation. Here we describe the parenchymal and non-parenchymal cell populations contributing to the tolerogenic status. An organ that represses immune reactions is a well-suited target for pathogens. Here we describe the viral pathogens targeting the liver, their infection behavior, and potential therapeutic options.

Keywords Hepatitis • Liver • Tolerance

1 Introduction

For several organs, such as gonads, the eye, or the central nervous system, a status as immune-privileged site has been well accepted. The molecular and cellular mechanisms determining immune privilege differ between these organs and are covered elsewhere within this book. Key to the function of an immune-privileged organ is the ability to locally skew immune responses to either prevent or escape

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induction as well as execution of antigen-specific immune responses. Typically, innate immune defense systems are conserved in these organs to assign the potential to fight infection by pathogens. Such innate immune function does not necessarily have to be carried out by bone marrow-derived immune cells but may be accomplished by organ-resident cells that are also equipped with the necessary immune sensory receptors, such as membrane-bound Toll-like receptors and cytosolic nucleic acid-sensing receptors. These receptors recognize conserved microbial patterns to induce innate immune defense such as induction of proinflammatory cytokines and effector molecules executing innate effector function like defensins. While the skewing of adaptive immune responses to prevent immunopathology to an organ, for example, prevention of scarring of the cornea to preserve vision, may be beneficial for the entire organism, such local immune regulation predisposes immune-privileged organs to the threat of infection.

Herein we will review the current knowledge on the cellular and molecular mechanisms that determine the status of the liver as an immune-privileged organ.

2 Evidence for Hepatic Immune Privilege

Studies in transplantation medicine have demonstrated that the liver has particular immune regulatory properties. Orthotopic liver transplants are well accepted for an extended period of time even with low doses of immunosuppression. In animal transplantation experiments, it was shown as early as in the 1960s that livers can be transplanted in pigs and will be accepted without immunosuppression (Calne et al. 1969), whereas other organs were rejected. It is noteworthy that in animals receiving liver transplantation together with another organ transplanted from the same donor, the liver provided protection for the second organ and led to graft acceptance without immunosuppression (Calne et al. 1969). This protective role of the liver in organ transplantation is not related to a general state of immunosuppression but is antigen-specific because transplantation of other organs from a third-party donor is rejected. This unique contribution of the liver to transplantation tolerance was also observed in mice (Qian et al. 1994), rats (Zimmermann et al. 1984), and dogs (Starzl et al. 1965). In humans, immunosuppression cannot be discontinued after liver transplantation to demonstrate the inherent tolerogenic function of the liver allograft, but data from patients who failed to take immunosuppressive drugs indicate that the human liver allografts can be accepted without or with insufficient immunosuppression. Similar to the observation in experimental organ transplantation in animals, transplantation of the liver in combination with a kidney allograft from the same donor induced tolerance towards the cotransplanted organ also in humans (Rasmussen et al. 1995). Similar to immune privilege at other organ sites, liver transplantation tolerance seems to be dominated by expression of death-inducing receptors such as FAS-L and the contribution of regulatory T cells (Watanabe et al. 2003; Watanabe et al. 2002). However, the mechanisms involved in the induction of liver allograft tolerance have not been entirely resolved.

The liver also fulfills a tolerogenic function towards circulating soluble antigens because of its metabolic function. It is situated between the systemic blood

circulation and the gastrointestinal tract. Blood is supplied by 25% of the hepatic artery and by 75% by venous blood from the portal vein, rich in nutrients but with low oxygen tension. Blood coming from the gut is not only rich in nutrients but it also carries degradation products from the gut, i.e., microbial products and antigens, like LPS, which act as pathogen-associated molecular pattern and are therefore ligands for pattern recognition receptors (PRR) expressed on hepatic cell populations (Lumsden et al. 1988). Although the liver is physiologically exposed to low concentrations of bacterial degradation products in portal venous blood (Jacob et al. 1977), there is no inflammation of the liver under these circumstances, which indicates that innate immune activation is locally controlled to prevent unnecessary inflammatory reactions towards innocuous material in portal blood. In fact, clearance of bacterial degradation products from the portal blood and immune sensing is mainly achieved by sinusoidal liver cells, i.e., liver sinusoidal endothelial cells (LSECs) and Kupffer cells that constitute the reticuloendothelial system of the liver. The local release of anti-inflammatory mediators such as IL-10 (Knolle et al. 1995), TGF-β (Bissell et al. 1995), and prostaglandins (Rieder et al. 1990) has been suggested to restrict such inflammatory reactions. Similarly, continuous presence of LPS may lead to a state of nonresponsiveness, termed endotoxin tolerance, that also provides cross-tolerance towards other proinflammatory stimuli elicited by stimulation through ligands of distinct Toll-like receptors or by hypoxic conditions (Biswas and Lopez-Collazo 2009). Interestingly, the scavenger function of LSECs and Kupffer cells is not impeded by their immune sensory function, indicating that the hepatic clearance function is not affected by local inflammatory reactions (Biswas and Lopez-Collazo 2009).

However, immune regulation in the liver is not restricted to control of innate immune reactions preventing unnecessary immunopathology but also entails the regulation of adaptive immune responses. In the following section, we will discuss the contribution of the different hepatic cell populations to local immune regulation in the liver.

3 Hepatic Cell Populations Mediating Liver Immune-Privilege

3.1 Hepatocytes

Hepatocytes are the parenchymal cells of the liver and therefore fulfill the tasks that are unique for the liver. This is metabolic function in carbohydrate, lipid, and protein turnover. But hepatocytes are becoming more into the focus of immunology. Hepatocytes express MHC class I molecules and Inter-Cellular Adhesion Molecule-1 (ICAM-1) (Chen et al. 2005; Bumgardner et al. 1990). Although separated from the bloodstream by LSEC, the unique property of LSEC, the fenestrae, allows the hepatocytes to establish direct access to lymphocytes circulating with the blood through hepatic sinusoids (Warren et al. 2006). Although

hepatocytes do not express costimulatory molecules, like CD80 and CD86, stimulation of naïve CD8+ T cells is achieved by hepatocytes and initially results in an identical phenotype and proliferation as stimulation by professional antigen-presenting cells (APCs) such as dendritic cells (DCs). Differences appear, however, in survival of CD8+ T cells stimulated by antigen-presenting hepatocytes. Whereas DC-stimulated CD8+ T cells survive for several days, CD8+ T cells stimulated by hepatocytes die after 3 days (Bertolino et al. 1995). This elimination of antigen-specific CD8+ T cells is accentuated by antigen-specific recruitment of naïve T cells to the liver depending on antigen presentation by hepatocytes (Bertolino et al. 2005). Importantly, initial antigen encounter of naïve T cells in the liver induces immune tolerance, whereas first antigen encounter in lymphatic tissue induces T cell immunity (Bowen et al. 2004), demonstrating that intrahepatic antigen presentation by tolerogenic hepatocytes has important consequences for skewing of adaptive T cell immunity. The mechanism underlying hepatocyte-mediated antigen-specific elimination of naïve T cells has recently been unraveled as a unique process of emperipolesis into hepatocytes followed by T cell degradation (Benseler et al. 2011).

Taken together, these findings underline a contribution of hepatocytes to the immune-privileged status of the liver.

3.2 Liver Sinusoidal Endothelial Cells

LSECs are cells lining the smallest hepatic vessels, the sinusoids equipped with unique properties. In contrast to most other endothelium, which functions as a barrier of cells interconnected by tight junctions and situated on a basal membrane separating the blood from parenchymal tissue, the endothelium of the liver sinusoids is fenestrated without an underlying basal membrane that allows direct contact of blood with hepatocytes. The fenestrae are holes within the LSEC with a diameter of approximately 100 nm, which can be dynamically regulated and are arranged in so-called sieve plates. Electron-lucent extracellular matrix produced by stellate cells within the space of Dissé presumably narrows the diameter of fenestrae. This reduces the actual size of particles passing through them to around 20 nm. This unique microanatomic architecture facilitates exchange of fluid and chylomicrons between the blood and the hepatocytes (Braet and Wisse 2002). LSECs form, together with the Kupffer cells, the so-called reticuloendothelial system of the liver. As a part of this, LSECs have an exceptional high scavenger function necessary for clearing the blood from bacterial degradation products and other antigens.

LSECs have unique functions as APCs. They express low levels of MHC class II molecules and costimulatory CD80/CD86, but the constant challenge with LPS leads to a downregulation of these molecules to residual surface expression (Knolle et al. 1999). In contrast, LSECs express high amounts of MHC class I molecules and are able to cross-present soluble molecules that have taken up by

receptor-mediated endocytosis (Limmer et al. 2000). As professional antigen-presenting cells, LSECs bear the capacity to prime naïve T cells for proliferation and cytokine expression. The outcome of cross-priming of CD8 T cells by LSEC, however, differs from priming by immunogenic DCs. LSEC-primed CD8 T cells lack cytotoxic effector function as well as the capability to produce effector cytokines like IFN-γ and TNF (Limmer et al. 2000; Diehl et al. 2008) and therefore were rendered tolerant in an antigen-specific fashion. However, in contrast to priming of naïve CD8 T cells by immature DCs under noninflammatory conditions, LSEC-primed CD8 T cells are not deleted. Although these primed T cells do not exert effector function, they do not undergo apoptosis but express high amount of the antiapoptotic molecule Bcl-2 (Diehl et al. 2008). Mechanistically, induction of tolerance by LSEC requires expression of the coinhibitory molecule B7-H1 acting on the inhibitory receptor PD1 expressed on T cells (Diehl et al. 2008). It is of interest to note that the development of T cell tolerance is a dynamic and well-balanced process between positive signaling through the T cell receptor and negative signaling through PD1 (Schurich et al. 2010). T cell tolerance induced by LSEC contributes to the unique immunologic function of the liver, where tolerance is induced towards blood-borne antigens.

In addition, LSECs use other mechanisms to prevent local immunogenic T cell priming in the liver. DCs that migrate through the liver as part of the normal blood–lymph transition pathway may initiate immunogenic T cell priming if they encounter appropriate inflammatory stimuli, which are present in portal venous blood. It was recently shown that LSECs veto the ability of immunogenic DCs to prime naïve T cells (Schildberg et al. 2008). This "veto effect" does not require antigen presentation by LSEC but direct cell–cell contact between LSEC and DC. The molecular mechanism is still under investigation. But by this mechanism, LSEC further contributes to the tolerogenic milieu in the liver.

Of note, the tolerance-inducing phenotype of LSEC can so far only be broken during viral infection of LSEC. Experimental infection of LSEC with a murine cytomegalovirus expressing the model antigen ovalbumin renders them capable to stimulate naïve CD8 T cells specific for ovalbumin (OT-I T cells) and lead to functional maturation into effector T cells (Kern et al. 2010). This phenotype strictly depends on live virus and is independent of the receptors recognizing pathogen-associated molecular patterns (PAMPs) because UV-inactivated virus, also triggering receptors for viral recognition, does not lead to a break of tolerance induction.

3.3 Kupffer Cells

Kupffer cells as liver-resident macrophages are the largest population of macrophages in the body. As member of the reticuloendothelial system, they are situated strategically inside the lumen of the sinusoids, where they can easily take up waste products, like senescent red blood cells, but also toxins and antigens from the circulation. Because of the narrow diameter of the sinusoids, they come in close

contact with circulating lymphocytes as NK, NKT, and T cells. Kupffer cells behave under noninflammatory conditions like tolerogenic APCs mediating tolerance towards antigens (Ju et al. 2003) but also in liver transplantation (Sato et al. 1996), thereby contributing to immune privilege of the liver. Mechanistically, they do not express MHC class II or costimulatory molecules, but they inhibit T cell activation by secretion of prostaglandins, like PGE_2 and 15-deoxy-delta12,14-PGJ_2 (15d-PGJ_2) (You et al. 2008). Inhibition of T cell immunity is also mediated by the activation of regulatory T cells (Treg) by Kupffer cells, leading to IL-10 production, which is necessary for tolerance induction of hepatocyte-presented antigens (Breous et al. 2009). Recently, a role for Kupffer cells in the attenuation of infection-induced immune damage of the liver was reported (Sitia et al. 2011; Lang et al. 2010). Taken together, also Kupffer cells combine scavenger function with regulatory immune function. However, their contribution to the development of local immunity against bacterial infection, for instance, by interacting with NKT cells in the hepatic sinusoid, has been demonstrated (Lee et al. 2010).

3.4 Hepatic Dendritic Cells

The liver harbors different kinds of dendritic cells (DCs). In mice the subsets comprise of plasmacytoid DCs ($CD11c^{low}$, B220+, CD11b−, LY6C+, SIGLECH+), myeloid DCs (CD11c+,CD11b+), and some smaller subsets, i.e., CD8+ DCs (CD8α+, CD11c+, CD11b−). In humans the main DC population are myeloid DCs (CD11c+, CD11b+, blood DC antigen-1 + (BDCA)) and plasmacytoid DCs (CD123+, BDCA2+, BDCA4+) (Bosma et al. 2006).

Upon stimulation with TLR ligands, myeloid DCs (mDCs) in the liver produce less IL-12 (Abe et al. 2006) but rather secrete IL-10 and IL-27 compared to mDCs from other organs like the spleen (Chen et al. 2009). This impaired function may be due to the continuous stimulation of DCs in the liver with PAMPs from the gut and helps to maintain the tolerogenic milieu in the liver. These PAMPs must not directly act on the DCs itself. LPS can stimulate hepatocytes to produce IL-6, which activates STAT3 (signal tranducer and activator of transcription 3) in DCs (Lunz et al. 2007). STAT3 activation in DCs induces expression of IRAK-M (IL-1 receptor-activating kinase-M), by itself a negative regulator of Toll-like receptor signaling. This may prevent maturation of DC upon stimulation and thereby renders DC in a state-mediating tolerance.

Also plasmacytoid DCs (pDCs) located in the liver are in a state different than that from other organs. In contrast to splenic DCs, they express lower levels of MHC class II and costimulatory molecules and by this are poor stimulators of T cells (Villadangos and Young 2008). Additionally, upon stimulation of liver-derived pDCs with cytomegalovirus (Jomantaite et al. 2004) or with CpG-containing DNA (Castellaneta et al. 2009), they produce less amounts of type I interferon compared to their counterparts in the spleen. Again, this lack of full DC activity may result from continuous stimulation with PAMPs derived from the gut.

3.5 Regulatory T Cells

Regulatory T cells have been reported to be involved in containing immune responses either by restricting the expansion of T cells or by inhibiting their effector function. Molecular mechanisms involved in their regulatory effector function are IL-10 and TGFβ, but they may also act by binding soluble IL-2, thereby depriving effector T cells of a critical growth and differentiation factor. However, in the liver, regulatory T cells are relatively scarce. There is evidence that LSECs induce an unusual regulatory T cell population that is negative for Foxp3 but retains its regulatory function on T cells (Kruse et al. 2009). Besides generation of regulatory T cells, their expansion seems to be promoted by LSECs as well as Kupffer cells (Wiegard et al. 2005). But also hepatocytes are involved in the induction of regulatory T cells. Expression of the neuronal autoantigen myelin basic protein (MBP) under the hepatocyte-specific CRP promoter or production of MBP in hepatocytes after adenoviral infection protected mice from neuronal inflammation induced by experimental autoimmune encephalomyelitis in a regulatory T cell-dependent manner (Luth et al. 2008). The most prominent mechanism for increasing the numbers of regulatory T cells in the liver, however, is their chemokine-driven recruitment. Regulatory T cells preferentially use the chemokine receptor CXCR3 and thereby use the same receptor as effector T cells homing to the liver (Oo et al. 2010). The induction of local inflammation in the liver thereby triggers chemokine-dependent simultaneous attraction of both effector and regulatory T cells. The consequences of this coincident recruitment for the outcome of hepatic immune responses, for example, against infecting pathogens, have not been properly addressed so far.

4 Viral Infection of the Liver

Given the unique immune regulatory properties of the liver, it is not astonishing that the liver is the target of infectious microorganisms. Although many pathogens reach the liver via the blood and are efficiently eliminated there, some pathogens specifically target the liver to establish infection. Viral infections of the liver, with hepatitis B or hepatitis C virus, occur in almost half of the world's population and become persistent in about 500 million people. The consequences of persistent hepatic infection can be both silent and clinically asymptomatic or can cause chronic inflammation leading to fibrosis and cirrhosis as well as hepatocellular carcinoma.

In principle, the liver is infected by many viruses. A few examples illustrating the clinically most relevant viruses are listed in Table 1.

Table 1 List of clinically relevant viral infections of the liver

Virus	Outcome of infection	Reference
HAV	Resolved after protracted infection	Lanford et al. (2011)
HBV	May be cleared after protracted infection or persist for years	Chisari et al. (2010)
HCV	May be cleared after protracted infection or persist for years	Bowen and Walker (2005)
HDV	May establish superinfection in combination with HBV infection	Rizzetto (2009)
HEV	Resolved after acute infection	Aggarwal and Jameel (2011)
EBV	May infect the liver during systemic infection	Petrova and Kamburov (2010)
CMV	May infect the liver during systemic infection	Gallegos-Orozco and Rakela-Brodner (2010)

4.1 Acute Infection

Viral infections may be spontaneously cleared by the immune system. The parameters relevant for such efficient antiviral immunity in the liver have been defined as rapid and multispecific CTL responses in combination with the generation of neutralizing antibodies against viral epitopes. This requires in both cases the efficient generation of CD4 T helper cells that support development of fully functional virus-specific CTLs as well as giving help for development of B cell differentiation (Rehermann and Nascimbeni 2005). An example for a virus efficiently cleared from the liver is the hepatitis A virus. Contrary to common belief, HAV infecting the liver is not cleared when virus titers in blood and feces become undetectable but persists for several weeks in hepatocytes (Lanford et al. 2011). This indicates that clearance of viruses from infected hepatocytes is a complicated process that requires efficient immunity and time. Furthermore, it is not entirely clear why HAV but not hepatitis C virus is cleared from the liver. HAV and HCV bear many resemblances as they are both RNA viruses and both inhibit innate immune sensing through the cytosolic nucleic-acid-specific receptor RIG-I (Gale and Foy 2005). Both viruses are capable of mutational immune escape and therefore in theory should show similar clinical courses. Further comparative studies are warranted to discover the relevant molecular mechanisms that determine whether viral infection is cleared or persists in infected hepatocytes.

4.2 Persistent Infection

Two hepatotropic viruses can persist in the liver, i.e., HBV and HCV. Both viruses target the liver by mechanisms that are still ill-defined. So far, we do not know any liver-specific or hepatocyte-specific receptor molecule that would explain the

extraordinary hepatotropism of these viruses. Instead, for HCV, several receptors have been identified that are involved in hepatocyte infection. These are CD81, scavenger receptor type I, and claudin, all molecules that show broad tissue expression (Pileri et al. 1998; Scarselli et al. 2002; Evans et al. 2007). For HBV, no clear candidate receptor on hepatocytes has been identified to date. This raised the question how blood-borne viruses efficiently target the liver. Accumulating data suggest that viruses do first target sinusoidal cells such as LSECs and Kupffer cells. HCV also binds to DC-SIGN and L-SIGN, although binding to these molecules does not initiate infection (Gardner et al. 2003; Pohlmann et al. 2003). These molecules are expressed on LSECs and Kupffer cells but not hepatocytes. Similarly, HBV in the duck model has been shown to first enter LSEC (Breiner et al. 2001). Collectively, these results suggest that viruses are first taken up by scavenger sinusoidal cell populations and then in a second step following a transcellular transport process infect neighboring hepatocytes, their final target cells.

It is important to note that both viruses employ strategies to escape innate immune detection, which is important for generation of protective immunity. HCV is detected in infected hepatocytes by RIG-I and TLR3 but prevents signaling from these pattern recognition receptors by interfering with the signaling adapter proteins IPS1/MAVS and IRF3 (Gale and Foy 2005; Foy et al. 2003; Saito et al. 2008), which disrupts induction of interferon or expression of interferon-sensitive genes that are important for innate antiviral defense. HBV employs another strategy to circumvent innate immune recognition. Following experimental HBV infection, there is little innate immune sensing in the liver that gives rise to expression of interferon. Yet, HBV infection does not go completely undetected because liver sinusoidal cell populations, i.e., LSECs and Kupffer cells, secrete IL-6 and IL-10 upon contact with HBV (Hosel et al. 2009). Kupffer cell-derived IL-10 has been shown to impede local immune responses in the liver (Knolle et al. 1998) and thus may also impair antiviral immunity against infected hepatocytes. Taken together, we can conclude that the liver is an organ that is capable of innate immune sensing and mount potent innate immune effector responses (Gao et al. 2008), but that hepatotropic viruses establishing persistent infection circumvent or blunt immune sensing in the liver. Such viral immune escape strategies may be involved in the pathogenesis of chronic liver infection.

Once chronic infection by HBV and HCV is established, there are continuous efforts of the immune system to clear infection from hepatocytes. Key to antiviral defense against viral infection are both antiviral cytokines such as TNF and interferons in combination with virus-specific cytotoxic T cells that eliminate infected hepatocytes after recognizing their target antigen presented in the context of MHC I molecules. The persistent attack of proinflammatory cytokines and cytotoxic T cells in the virus-infected liver leads to chronic liver inflammation. While acute inflammation and accompanying intermediate levels of hepatocyte death are well tolerated by the liver because of its extraordinary capacity to regenerate, long-lasting inflammation is known to lead to development of liver fibrosis (Guidotti and Chisari 2006). Eventually, when chronic inflammation persists, bridging fibrosis develops in inflamed liver tissue that results in scarring

and a loss of liver function. The endpoint of this development is liver cirrhosis, which carries a bad prognosis because of its manifold complications such as portal hypertension causing life-threatening bleeding from esophageal varices or ascites formation that is further complicated by spontaneous bacterial peritonitis.

Key to the inability of the immune system to clear HBV and HCV from infected hepatocytes are not only viral immune escape mechanisms to circumvent T cell- and antibody-mediated immunity. Inflammation also upregulates coinhibitory receptors such as B7H1 on liver cells that impede the execution of effector function of virus-specific cytotoxic T cells once they entered the liver (Zhang et al. 2008; Kassel et al. 2009). As B7H1 is expressed on Kupffer cells, LSECs, stellate cells, and hepatocytes T cells are continuously exposed to inhibitory signaling once they arrive in the liver. Such inhibitory signaling is crucial for protection of the liver from inadvertent immune reactions because loss of B7H1 expression on liver but not immune cells causes the development of liver inflammation presumably as a consequence of organ autoimmunity (Dong et al. 2004; Iwai et al. 2003). While expression of coinhibitory receptors may be advantageous in preventing overzealous immune reactivity and immunopathology, it may at the same time limit virus-specific T cell immunity. Thus, we can conclude that both viral immune escape strategies as well as liver-specific immune regulatory cues contribute to the development of persistent viral infection of the liver.

4.3 Therapeutic Implications

The current mainstay of therapy for chronic viral infection of the liver is the combinatorial application of virus-specific antivirals together with type I interferon (Rosen 2011). For HCV, the response rates for combinatorial treatment are very promising, which is likely to be related to the strict dependence of positive strand RNA viruses on continuous replication in order to persist. The situation is different for chronic hepatitis B, where response rates to combinatorial therapy are much lower (Werle-Lapostolle et al. 2004). While careful and systematic analysis of immune responses in patients with resolved compared to chronic hepatitis B disclosed that a potent cytotoxic T cell response is required for viral clearance, there is also evidence that strong virus-specific T cell responses overcome chronic infection. However, there are little if any HBV-specific cytotoxic T cells found in patients suffering from chronic hepatitis B, which may be attributed to exhaustion of T cells as described above (Lopes et al. 2008; Radziewicz et al. 2008). Consistent with the few HBV-specific T cells found in hepatitis B patients, there are no promising reports on the development of therapeutic vaccination protocols to overcome persistent HBV infection. Based on our new and improved knowledge on the hepatic mechanisms blunting T cell responses in the liver, future strategies for immunotherapy will have to attempt to overcome inhibition of T cell function in the liver. Nevertheless, such approaches must be accompanied by efforts to increase the number of functional virus-specific cytotoxic T cells and their recruitment to the

liver. However, even if such achievements can be made, one has to pay particular attention to the danger of overwhelming immunopathology to the liver that may arise from such an approach. As outlined above, the immune-privileged organs are equipped with potent immune regulatory mechanisms to prevent immunopathology and preserve proper organ function. Deliberate inhibition of these functions, even with a well-taken therapeutic aim, may pose the organ to deleterious effects of overwhelming immunity.

Collectively, the unique immune regulatory function of the liver likely predisposes to development of chronic infection. While overcoming of inhibitory immune signaling may be key to a successful immune therapy against chronic viral infection in the liver, it may at the same time deprive the liver of an important protective shield that may lead to immune-mediated liver failure. Future development of immunotherapy will have to take into account this complex interaction to invent immune effector principles that can be rapidly reverted or neutralized once immunopathology gets out of control.

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Immune Homeostasis of the Lung: The Role of Regulatory NKT Cells in Asthma

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Abstract Bronchial asthma, a chronic inflammatory disease of the lungs, is a complex and heterogenous disease that has increased dramatically in prevalence over the past 3 decades. The increasing prevalence of asthma is thought to be due to changes in our environment, particularly to reductions in the incidence of several infectious diseases that exert protective effects against asthma, as suggested by the Hygiene Hypothesis. Accumulating studies suggest that exposure to a variety of microbiota affects the immune system, resulting in protection against the development of asthma and allergy. However, the specific immunological mechanisms responsible for the inverse relationship between infections and asthma are far from clear. In this review, we focus on immune homeostasis in the lungs in the regulation of asthma. We discuss the classical paradigm of Th2 cells and allergy, but focus on control by regulatory T (Treg) cells and by a novel regulatory immune cell type—a suppressive subset of natural killer T (NKT) cells. In addition, we discuss possible mechanisms for Hygiene Hypothesis by which infectious microorganisms protect against asthma, in which innate immune, rather than adaptive immune, cells may interact with microbes, including influenza A virus and *Helicobacter pylori*, and manipulate and shape immunity in young children. Finally, the understanding of the mechanisms that underlie the Hygiene Hypothesis may result in new therapeutic approaches to the prevention of these disorders.

Keywords Asthma • Hygiene Hypothesis • NKT • Treg

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1 Introduction

The prevalence of asthma, a major public health problem affecting 300 million people worldwide, has increased dramatically over the past 3 decades. This increase is thought to be due to changes in our environment, such as increases in air pollution, increases in the prevalence of obesity, increased use of acetaminophen, as well as reductions in the incidence of some infectious diseases that exert protective effects against asthma, as suggested by the Hygiene Hypothesis (Strachan 1989). While the Hygiene Hypothesis has received a great deal of attention for more than 20 years, surprisingly, only a few infectious microorganisms have been identified to account for this relationship. These microbes include *Helicobacter pylori* (Matsuda et al. 2000; Reibman et al. 2008) and the hepatitis A virus (HAV) (Matricardi et al. 1997, 2002). On the other hand, it is possible that a diverse collection of commensal bacteria together may be responsible for protection against asthma and allergy (Ege et al. 2011). Nevertheless, the mechanisms by which microbes control the development of asthma and allergy are largely unknown. However, understanding of these regulatory mechanisms is likely to provide profound insight into the pathophysiology of asthma and allergy. In this review, we will discuss immune homeostasis in the lungs in the regulation of asthma. We will discuss the classical paradigm of Th2 cells and allergy, but focus on its suppression by regulatory T (Treg) cells and by novel regulatory immune cells such as subsets of natural killer T (NKT) cells. In addition, we will discuss possible innate mechanisms that mediate the Hygiene Hypothesis and how infectious microorganisms might protect against asthma.

2 Asthma

2.1 Clinical Features, Immune Mechanisms

Asthma is a complex trait caused by multiple environmental factors in combination with more than 100 major and minor susceptibility genes (Umetsu et al. 2002; von Mutius 2009). The dramatic rise in the prevalence of asthma and allergy over the past 30 years is due to changes in the environment and not to changes in the genetic composition of the population and presents an important puzzle in immune regulation. In this puzzle, environmental events that were common 30 years ago suppress the development of asthma and allergy by affecting various components of the immune system. As suggested by Strachan, and articulated in the Hygiene Hypothesis (Strachan 1989), the solution to this puzzle involves identification of the infections that most likely affect the immune system and that protect against asthma and allergy. Strachan noted that children in larger families were less likely to develop asthma and allergies. In addition, others have noted that early placement in daycare settings (Ball et al. 2000), or exposure to farming environments (Riedler

et al. 2001; Braun-Fahrlander et al. 2002), or dogs or cats (Ownby et al. 2002) in early life protected against the development of asthma and allergies. The interpretation of these observations is that infection, acquired from siblings and in the daycare setting (particularly HAV (Hadler et al. 1980)), could protect against asthma and allergy. In farming environments, exposure to microbiota may substitute for respiratory or gastrointestinal infections. Indeed, farming environments have been shown to include high levels of endotoxin, associated inversely with atopic wheeze (Braun-Fahrlander et al. 2002). Furthermore, a Gram-negative, nonpathogenic bacterium *Acinetobacter lwoffii* F78 has been isolated from the cowshed dust samples and has been shown in mouse models to protect against the development of allergen-induced airway disease (Debarry et al. 2007). Moreover, farming environments with higher diversity of microbiota were associated with better protection against asthma (Ege et al. 2011). These observations suggested that exposure to a wide range of microbiota affects the developing immune system, which then prevents the development of asthma and allergy. However, the specific immunological mechanisms responsible for the inverse relationship between infections and asthma are far from clear. Presumably, exposure to microbiota affects the development of regulatory/suppressive cells, but few studies have provided support for this possibility. Identification of these infectious agents and immune responses, especially the impact for lung immunity, are likely to provide important insight into the immune mechanisms and regulatory immune responses that protect against the development of asthma and allergies.

Allergic asthma is the most common form of asthma and is characterized by symptoms of intermittent wheezing and airway inflammation, associated with eosinophils, basophils, lymphocytes, and increased mucus production. Allergy has been extensively studied over the past 20 years as a major risk factor for persistent asthma and is responsible for many of the classical features of asthma. Allergic asthma is mediated by adaptive immunity and Th2 cells, and virtually all patients with asthma are thought to have allergen-specific Th2 cells in their airways (Robinson et al. 1992). Th2 cells orchestrate the development of asthma by secreting Th2 cytokines, which enhance the production of allergen-specific IgE (IL-4), promoting the growth of eosinophils (IL-5) and mast cells (IL-9), and by directly causing airway hyperreactivity (AHR) (IL-13), a cardinal feature of asthma. In addition, the effects of allergen-specific Th2 cells in the airway may be amplified by $CD4^+$ natural killer T (NKT) cells (Akbari et al. 2006), which rapidly produce very large amounts of IL-4, IL-5, and IL-13; which are required for the development of allergen-induced AHR in mice (Akbari et al. 2003); and which are present in the lungs of patients with severe asthma (Akbari et al. 2006).

Over the past 5 years, however, it has become increasingly apparent that asthma is more heterogeneous and complex than suggested by the Th2 paradigm and that asthma symptoms are often associated with many nonallergic factors. For example, nonallergic forms of asthma, triggered by environmental factors, such as air pollutants (e.g., smoke, diesel particles, and ozone) (Pichavant et al. 2008; Robays et al. 2009; Li et al. 2003), stress, obesity (Johnston et al. 2007), and viral infection and severe steroid-resistant asthma (Wang et al. 2010), develop regardless of the

presence of allergy, presumably independent of Th2 cells (Pichavant et al. 2008; Johnston et al. 2007; Kim et al. 2008; Wright 2005). Furthermore, non-Th2 factors such as IFN-γ and IL-17 are frequently found in the lungs of patients with asthma, particularly in the lungs of patients with severe or steroid-resistant asthma. In many of such patients, neutrophils, rather than eosinophils and Th2 cells, predominate. Therefore, other cell types and processes, in addition to Th2 cells, must regulate the development of asthma.

2.2 Immune Mechanisms in Asthma

A critical feature of all adaptive immune responses, including allergic responses, is the regulation by a cadre of T cells called regulatory T (Treg) cells having regulatory or suppressive activity. These include Foxp3 Treg cells, T_R1 cells, Th3 cells, and several additional novel cell types, such as iTR35 cells, which are described below, as they relate to asthma.

2.2.1 Foxp3 Expressing Antigen-Specific Treg Cells

A key role for Foxp3 expressing Treg cells in preventing autoimmune disease and allergy is illustrated by patients with deleterious mutations in the Foxp3 gene, who develop severe autoimmune disease and allergy in infancy (Bennett et al. 2001). Foxp3 expressing Treg cells can be divided into "natural" Treg cells, which develop in the thymus with specificity for autoantigens and which prevent autoimmune disease, as well as "induced" Treg cells, which develop in the periphery on exposure to exogenous antigen or allergen. Treg cells express CTLA4 and produce IL-10, TGF-β, and IL-35 (Roncarolo et al. 2006; Chen et al. 2003; Collison et al. 2007). The distinction between natural and induced Treg cells has been vague but has been recently substantiated by studies demonstrating that natural, but not induced, Treg cells express the Helios transcription factor (Verhagen and Wraith 2010; Getnet et al. 2010; Thornton et al. 2010), and studies showing that deletion of the CNS3 region in the Foxp3 promoter eliminates the development of natural Treg cells in the thymus, whereas deletion of the CNS1 region in the Foxp3 promoter affects only induced Treg cell development in the periphery (Zheng et al. 2010). Importantly, the development of induced Treg cells was greatly reduced in mice housed in germ-free conditions but restored by recolonization of the mice with *Clostridium* species (Atarashi et al. 2011). The effect of the *Clostridium* species was specific for adaptive Treg cells, since another spore-forming Gram+ bacteria, segmented filamentous bacteria, specifically induced Th17 cells (Atarashi et al. 2011; Ivanov et al. 2009).

In allergy, patients are thought to have a deficiency in the number or function of allergen-specific Treg cells and an overabundance of allergen-specific Th2 cells (Akdis et al. 2003). Allergen immunotherapy for allergic rhinitis and asthma,

performed by administration of increasing amounts of allergen, is thought to enhance the development of IL-10 producing Treg cells (Meiler et al. 2008; Akdis et al. 2004; Verhoef et al. 2005). In murine models, both natural and allergen-specific induced Foxp3-expressing Treg cells inhibit the development of AHR, although induced allergen-specific Treg cells may be more efficient in these experimental models of asthma (Akbari et al. 2001; Stock et al. 2004). Induced Treg cells that develop in the context of respiratory exposure to allergen express IL-10, GATA3, and Foxp3 (Akbari et al. 2001), while those that develop on immunization with heat killed *Listeria monocytogenes* as adjuvant express T-bet, IFN-γ, IL-10, and Foxp3 (Stock et al. 2004), suggesting that a spectrum of Treg cell types may exist, depending on the inducing conditions. Furthermore, treatment of mice with *Clostridium* species, which increased the development of induced Treg cells, not only reduced symptoms of colitis induced with dextran sulfate but also reduced the production of allergen-specific IgE (Atarashi et al. 2011). This suggests that host commensal microbiota have important effects on the development of induced Tregs and the development of allergy.

2.2.2 T_R1 and Th3 Cells

Another type of regulatory T cell called regulatory T_R1 cells produces IL-10 and IL-5 but not IL-4 and do not express Foxp3 (Groux et al. 1997). Treg cells, induced by exposing antigen-specific T cells to IL-10, have been implicated in prevention of graft-versus-host disease and in experimental colitis and in preventing the development of allergy (Meiler et al. 2008). T_R1-like cells specific for bee venom were found in beekeepers, increased rapidly in number in the spring, and diminished in number during the winter months. This correlated inversely with the proliferative response of peripheral blood T cells to bee venom (Meiler et al. 2008). T_R1 cells may be particularly lacking in patients with corticosteroid-resistant asthma in association with vitamin D deficiency (Xystrakis et al. 2006). A third type of regulatory T cell, called Th3 cell, produces transforming growth factor-β (TGF-β) and varying amounts of IL-4 and IL-10 and has been described primarily in the context of oral antigen exposure, in mesenteric lymph nodes (Chen et al. 1994). Such Th3 cells have been shown to inhibit the development of EAE.

2.2.3 iTR35 Cells

IL-35 is a Treg cell-specific cytokine that is required for the maximum regulatory activity of mouse Treg cells in vitro and in vivo (Collison et al. 2007, 2010). IL-35 is a heterodimer, consisting of Epstein–Barr-virus-induced gene 3 (Ebi3, which encodes IL-27β) and interleukin-12 alpha (Il12α, which encodes IL-12a/p35). In an inflammatory bowel disease (IBD) model, IL-35 was found to be highly expressed by mouse $Foxp3^+$ Treg cells but not by resting or activated effector $CD4^+$ T (Teff) cells, and that an Ebi3–IL-12α heterodimer is constitutively secreted

by Treg but not Teff cells (Collison et al. 2007). In addition, both Ebi3 and Il12α messenger RNA are markedly upregulated in Treg cells cocultured with Teff cells, thereby boosting Ebi3 and IL-12α production in trans. Furthermore, Treg cells generated from $Ebi3^{-/-}$ and $Il12a^{-/-}$ mice have significantly reduced regulatory activity in vitro and fail to control homeostatic proliferation and to cure inflammatory bowel disease in vivo (Collison et al. 2007). Because these phenotypic characteristics are distinct from those of other IL-12 family members, this novel Ebi3–IL-12a heterodimeric cytokine has been designated interleukin-35 (IL-35). Ectopic expression of IL-35 confers regulatory activity on naïve T cells, whereas recombinant IL-35 suppresses T-cell proliferation (Collison et al. 2007). Taken together, these data identify IL-35 as a novel inhibitory cytokine that may be specifically produced by Treg cells and is required for maximal suppressive activity.

Collison et al. further referred to this iT_R population as "iTR35 cells," which was induced by treatment of naïve human or mouse T cells with IL-35 and mediated suppression via IL-35 but not via the inhibitory cytokines IL-10 or TGF-β. They showed that unlike the other iT_R populations described before, TGFβ iT_R and IL-10–iT_R cells, which require longer conversion protocols, multiple cell types, and/or additional molecules for optimal generation (Groux et al. 1997; Barrat et al. 2002; Kemper et al. 2003), iTR35 cells were generated by a single, short-term stimulation of the T-cell antigen receptor in the presence of IL-35 (mouse, 3 days; human, 6 days) (Collison et al. 2010). In the in vitro studies, IL-35 was shown to convert human T_{conv} cells into a homogeneous population of iT_R cells, while in treatment of T_{conv} cells with mouse, IL-35 converted them into an IL-35-producing suppressive population (Collison et al. 2010). In an autoimmune disease model, mice treated with iTR35 cells were completely protected from EAE, whereas mice that received iTR35 cells from $Ebi3^{-/-}$ were indistinguishable from control mice that received saline, which suggested that IL-35 production by iTR35 cells in vivo is required for this protection (Collison et al. 2010). Furthermore, Treg cells induced the generation of iTR35 cells in an IL-35- and IL-10-dependent manner in vitro and induced their generation in vivo under inflammatory conditions in intestines infected with *Trichuris muris* and within the tumor microenvironment (B16 melanoma and MC38 colorectal adenocarcinoma), where they contributed to the regulatory milieu. In addition, iTR35 cells were also able to cure IBD induced by CD4-dnTGFβRII T_{conv} cells to further demonstrate that TGF-β is not required for the in vivo suppressive ability of iTR35 (Collison et al. 2010). Taken together, iT_R35 cells have a potent suppressive ability in a wide variety of in vivo autoimmune disease models, suggesting their potential role in allergy and asthma.

2.3 *Inflammatory and Suppressive NKT Subsets in Asthma*

In addition to regulation by T cells, asthma is also regulated by natural killer T (NKT) cells. In asthma, NKT cells have been shown to play a very important pro-inflammatory role (Akbari 2003; Lisbonne et al. 2003). For example, in models

of allergic asthma, $CD4^+$ NKT cells expressing IL-17RB (IL-25R) producing IL-4, IL-5, and IL-13 are required for the development of allergen-induced AHR. In contrast, in a model of air pollution-associated asthma, a double negative (DN) $NK1.1^-$ NKT cell subset producing IL-17 was required (Pichavant et al. 2008), and in a model of Sendai virus-induced chronic asthma, DN NKT cells producing IL-13 were required for AHR (Kim et al. 2008). However, recently a DN NKT cell subset producing IFN-γ has also been shown to play a very important *suppressive* role in the development of allergen-induced AHR (Chang et al. 2011).

The suppressive DN NKT cells were identified when 2-week-old pups were infected with influenza A virus, resulting in protection of the mice as adults against the development of allergen-induced AHR. In humans, although respiratory viral infection including influenza almost always causes asthma exacerbations in patients with established asthma (Glezen et al. 2000; Miller et al. 2008; Jain et al. 2009), viral infection in young children may protect against the later development of asthma (Tsitoura et al. 2000; Dahl et al. 2004; Marsland et al. 2004; Wohlleben et al. 2003). In our studies in mice, influenza A virus H3N1 infection in 2-week-old suckling mice indeed protected the mice as adults against allergen-induced AHR (Chang et al. 2011). In contrast, infection of adult (8-week-old) mice with the H3N1 virus did not protect against the subsequent development of AHR (Chang et al. 2011). These observations suggest that the timing of infection determines its effect on lung immunity and that a narrow window of opportunity exists for the protective effects of infection, either prenatally and/or early in life (Ege et al. 2006; Rowe et al. 2007).

The protective effect of infection in humans was initially thought to primarily affect adaptive immunity (North 1973; Blanden 1974; Niklasson and Williams 1974). However, epidemiological studies indicate that both Th2-mediated diseases and Th1/Th17-mediated diseases (inflammatory bowel disease, type 1 diabetes, and multiple sclerosis) have increased in prevalence over the past 30 years, suggesting that infections likely affect innate immune mechanisms that inhibit both Th1 and Th2 cell development. Indeed, the protective effect of H3N1 in suckling mice was associated with the maturation and expansion of an innate-like cell, a specific subset of NKT cells. These NKT cells, a $CD4^-CD8^-$ (double negative, DN), IFN-γ-producing NKT cell subset, were shown to suppress the development of allergen-induced AHR, when adoptively transferred to allergen-sensitized adult mice (Chang et al. 2011). The protective NKT cell subset required T-bet for development, as T-$bet^{-/-}$ mice, when infected at 2 weeks of age, were not protected against the later development of AHR. Furthermore, NKT cells from T-$bet^{-/-}$ mice infected at 2 weeks of age with influenza virus could not transfer protection. The suppressive effect of the DN NKT cells was mediated by $Foxp3^+$ Treg cells, since adoptive transfer of the protective NKT cell population was associated with the expansion of allergen-specific $Foxp3^+$ Treg cells (Chang et al. 2011). Moreover, the protective effect was associated with the preferential expansion of suppressive in lung and which is Toll-like receptor 7 (TLR7)-dependent (Chang et al. 2011). This suggested the innate immune cells, rather than adaptive immune cells, may interact with microbe infections and manipulate and shape the lung immunity in the early life, probably young children.

2.3.1 *Helicobacter pylori* Glycolipids

Although influenza virus affects many different cell types, NKT cells mediated the protective effect of H3N1 infection, since protection was not observed in NKT-cell-deficient mice infected at 2 weeks of age, and because the protective effect could be replicated by treating suckling mice with glycolipids that specifically activated NKT cells. NKT-cell-activating glycolipids from *H. pylori* (PI57) or α-C-GalCer, which is a known synthetic Th1-biased α-GalCer analog, when administered to suckling mice, protected the mice from the later development of allergen-induced AHR. Of note, this glycolipid, PI57, a synthetic analog of cholesteryl-6-*O*-acyl a-glucoside unique glycolipid, which was first isolated from the cell walls of *H. pylori* in 1995 (Hirai et al. 1995), could stimulate these suppressive NKT cells (Chang et al. 2011). Moreover, adoptive transfer of NKT cells from PI57-treated, but not vehicle-treated, 2-week-old mice (harvested 6 weeks after treatment) into OVA-sensitized WT mice suppressed AHR and airway inflammation. Transfer of NKT cells from α-GalCer-treated mice reduced AHR slightly, but this was not statistically significant. Furthermore, the production of IFN-γ by the NKT cells was important, since the protective effect of PI57, like that of influenza A virus H3N1, was dependent on T-bet, since PI57 treatment of 2-week-old $Tbet^{-/-}$ mice did not protect against subsequent OVA-induced AHR (Chang et al. 2011). In addition, PI57 has also been demonstrated as a CD1d-dependent NKT cell antigen with several in vitro or cell-free experiments (Chang et al. 2011). To demonstrate that PI57 can directly activate NKT cells, Chang et al. showed that PI57, when added to cultures of NKT cell lines plus DCs, induced the production of IFN-γ in a CD1d-restricted manner, since cytokine production was blocked by anti-CD1d mAb. In addition, PI57 induced higher levels of IFN-γ and less IL-4 in NKT cell lines compared with PBS30 (from Sphingomonas) or α-GalCer and did so in a CD1d-restricted manner, since DCs from $Cd1d^{-/-}$ mice failed to support PI57-induced cytokine production. Furthermore, the PI57 response occurred by direct activation of NKT cells, since PI57 induced cytokine production in NKT cell lines with DCs from $Myd88^{-/-}$ or $Trif^{-/-}$ mice and since 3 different NKT cell hybridomas derived from $V\alpha14^{+}$ NKT cells but not from $V\alpha14^{-}$ T cells produced IL-2 in response to immobilized recombinant CD1d previously loaded with PI57 but not with PI56, a control glycolipid (Chang et al. 2011). Moreover, CD1d tetramers loaded with PI57 stained 10–23% of NKT cells in an NKT cell line. Of the PI57-CD1d tetramer + cells, 92% were $CD4^{-}$ (DN). This strongly suggests that PI57 bound to CD1d was directly recognized by the TCR of a population of NKT cells. Finally, human NKT cells were also activated by PI57, since NKT cells lines as well as a $V\alpha24^{+}$ NKT cell clone responded to this glycolipid. The response was also directly induced, since plate-bound CD1d loaded with PI57 induced IFN-γ in BM2a.3 cells. Taken together, these results indicated that both mouse and human NKT cells were directly activated by PI57, an *H. pylori* glycolipid, in a CD1d-restricted manner (Chang et al. 2011). Taken together, these results together suggest

that a subset of suppressive NKT cells that can be specifically activated by certain glycolipid antigens, and that preferentially produces IFN-γ, mediates the protective effects on asthma.

These studies are particularly important not only because they characterize an NKT cell population that suppresses AHR but also because they provide a plausible mechanism for epidemiological studies showing that infection with *H. pylori* protects against the development of asthma (Reibman et al. 2008). Until now, however, the mechanisms of how *H. pylori* might affect asthma were not known. The expansion of a suppressive DN NKT cell population by the *H. pylori* glycolipid in mice suggests that *H. pylori* infection in humans may protect against asthma by expanding a similar suppressive NKT cell population in children.

3 Regulatory NKT Cells

The "regulatory" NKT cells mediating the inhibitory effect of H3N1 influenza infection on asthma may be similar to NKT cells previously described in several immune regulatory responses such as anterior chamber-associated immune deviation (ACAID)(Sonoda et al. 1999), inhibition of autoimmune disease (Taniguchi et al. 2003; Kronenberg 2005), transplant tolerance (Seino et al. 2001; Ikehara et al. 2000; Sonoda et al. 2002; Jiang et al. 2005), airway disease induced with IL-33, and possible to suppressive effects in tumor immunity (Berzofsky and Terabe 2008a, b; Terabe and Berzofsky 2008).

3.1 NKT Cells Required for ACAID

NKT cells play an important role in mediating anterior chamber-associated immune deviation (ACAID), which is a well-studied model of immune privilege and tolerance (Kaplan et al. 1975). In this model, introduction of antigen into the anterior chamber of the eye results in systemic tolerance. The process is initiated by $F4/80^+$ antigen-presenting cells (APC), which migrate and accumulate in the marginal zone (MZ) of the spleen, where they interact through CD1d molecule with iNKT cells (Sonoda et al. 1999). However, unlike the suppressive NKT subsets in the influenza model (Chang et al. 2011), in which double negative ($CD4^-$ $CD8^-$) produce IFN-γ (Chang et al. 2011), the iNKT cells required for ACAID expresses CD4 (Nakamura et al. 2003) and produces the immunosuppressive molecules IL-10 and TGF-β, (Sonoda et al. 1999, 2001) the chemokine RANTES (Faunce and Stein-Streilein 2002), and the serine protease urokinase (Sonoda et al. 2007). Since ACAID can be induced in MHC class $II^{-/-}$ but in NKT-cell-deficient $CD1d^{-/-}$ mice, and since antibodies to CD4 protein remove the ability to induce ACAID in these mice (Nakamura et al. 2003), $CD4^+$ iNKT cells, but not conventional $CD4^+$ T cells, are required in this process. The actual function of the CD4 molecule on the iNKT cell in ACAID is unknown.

3.2 NKT–pDC Cooperation in Diabetes

In a mouse model of type 1 diabetes, iNKT cells have been shown to inhibit the development of diabetes by impairing the differentiation of T helper 1 (Th1) anti-islet T cells and/or by recruiting tolerogenic myeloid dendritic cells (Beaudoin et al. 2002; Chen et al. 2005; Naumov et al. 2001). In this model, LCMV infection activates anti-islet T cells, which induces diabetes (Berzofsky and Terabe 2008b). A critical role of OX40–OX40L interaction in the enhancement of pDC functions by iNKT cells has shown in vivo (Diana et al. 2009). At steady state, OX40 was strongly expressed on iNKT cells from the pancreas and liver but not from lymphoid organs, such as the spleen and PLN. After LCMV infection, a significant increase in OX40 expression was found on those iNKT cells from the pancreas and liver, whereas this expression remained low in both the spleen and PLN (Diana et al. 2009). The critical role of OX40-expressing iNKT cells was further confirmed by cell-transfer experiments. Transfer of WT iNKT cells into OX40-deficient mice led to lower pancreatic viral load than transfer with OX40-deficient iNKT cells. Moreover, in vitro experiments with sorted iNKT cells from the pancreas or spleen and sorted pDCs showed that only pancreatic iNKT cells were able to interact with pDCs and to induce type I IFN production upon LCMV infection (Diana et al. 2009). Finally, blocking experiments, in vivo and in vitro, with OX40L mAb and OX40-Ig clearly demonstrated that the OX40–OX40L pathway was necessary for efficient iNKT cell–pDC cooperation (Diana et al. 2009). The iNKT cell–pDC interaction might induce reverse signaling through OX40L, as previously described in human DCs (Ohshima et al. 1997) and other cell types, such as B cells and vascular endothelial cells (Kotani et al. 2002; Stuber et al. 1995). Alternatively, pDC–iNKT cell interaction could lead to OX40-mediated activation of iNKT cells (Marschner et al. 2005; Montoya et al. 2006; Zaini et al. 2007), and this could, in turn, have positive feedback effect on pDCs. Recently, it was shown that these NKT-cell-modified pDCs in pancreatic LNs could convert naïve anti-islet T cells into $Foxp3^+$ $CD4^+$ Treg cells, which then produce TGF-β and dampen the islet-specific $CD8^+$ T cells preventing diabetes (Diana et al. 2011). Thus, these studies identify pDCs as an essential partner of iNKT cells to suppress the immune response and the development of type 1 diabetes and may reflect the protective role of IL-4-producing NKT cells proposed for human type I diabetes (Wilson et al. 1998).

3.3 NKT in Cardiac Transplantation

In several models for organ transplantation, NKT cells have also been shown to mediate a protective role. For example, Ikehara et al. (2000) demonstrated that NKT cells are essential for the acceptance of xenogeneic islets treated with anti-CD4 mAb, and Sonoda et al. (2002) reported that the long-term survival of corneal

allografts is dependent on NKT cells. In addition, NKT cells are required for the maintenance of transplant allograft tolerance, mediated by costimulation blockade (Seino et al. 2001). In a cardiac transplant model, NKT cells in cardiac recipients expressed higher IL-10 production in tolerant mice previously treated with CD40L blockade (Jiang et al. 2007). Moreover, adoptive transfer of an NKT-cell-enriched cell population from the WT mice, which was not observed in the IL-$10^{-/-}$ mice, could reverse the shortened survival time in the NKT-deficient recipients (Jiang et al. 2007). Furthermore, in the tolerant recipients, IL-10-producing regulatory DCs and CD4 T cells were also induced in an NKT-cell-dependent manner (Jiang et al. 2007). Moreover, this IL-10 production by NKT cells was essential for generating regulatory dendritic cells that produce higher levels of IL-10 and reduced levels of IL-12 (Kojo et al. 2005). Taken together, these data indicate the existence of IL-10-dependent immune regulatory interplay among NKT cells, DCs, and CD4 T cells, even in the absence of artificial stimulation of NKT cells with synthetic glycolipids, which is required for the maintenance of transplant tolerance (Jiang et al. 2007).

3.4 NKT in Bone Marrow Transplantation (IL-4)

NKT cells have also been shown to be essential in mediating tolerance in graft-versus-host disease (GVHD) (Zeng et al. 1999a, b, 2002; Lan et al. 2001). In bone marrow transplantation, $CD4^{-}CD8^{-}$ NKT cells inhibited GVHD (Zeng et al. 1999b; Palathumpat et al. 1992a). Depletion of NKT cells from the sorted marrow T cells or use of sorted marrow T cells from IL-$4^{-/-}$ donors resulted in lethal GVHD (Zeng et al. 1999b). Sorted donor IL-$4^{-/-}$ NKT cells that secreted high levels of IFN-γ without IL-4 exacerbated histopathological changes of GVHD and overall GVHD mortality when added back to donor marrow T cells depleted of NKT cells. The add-back of WT NKT cells ameliorated GVHD (Zeng et al. 1999b). Results showed that $CD4^{-}CD8^{-}$ NKT cells potently suppress acute lethal GVHD in an IL-4-dependent manner (Palathumpat et al. 1992b; Strober et al. 1996). In a model of total lymphoid irradiation (TLI) preconditioning of the recipient, TLI increased in the percentage of NKT cells as the number of TLI doses increased (Lan et al. 2001, 2003), from 1% in unirradiated controls to 60–70% among the residual T cells. Addition of antithymocyte serum (ATS) or antithymocyte globulin (ATG) to the irradiation regimen resulted in a further increase in the percentage of NKT cells to 490% of all $TCR\beta^{+}$ cells (Lan et al. 2001). The survival of the WT hosts was significantly improved compared with that of CD1$d^{-/-}$ or IL-$4^{-/-}$hosts, indicating that host CD1d-reactive NKT cells that secrete IL-4 contributed to protection against GVHD as compared with WT T cells (Lan et al. 2001, 2003). Sorted T cells from IL-$4^{-/-}$ mice given TLI failed to inhibit GVHD and worsened the survival of BALB/c hosts (Lan et al. 2001, 2003). Taken together, these experiments demonstrate the IL-4-dependent GVHD-suppressive capacity of both host- and donor-type regulatory NKT cells on donor GVHD-effector T cells after

allotransplantation. The interaction of NKT cells and Treg cells has also been reported in the GVHD (Kohrt et al. 2010). An important influence of host NKT cells and host IL-4 on the accumulation of donor Treg in the TLI/ATS-conditioned host spleen 6 days after transplantation was observed. Donor Treg division and accumulation were markedly reduced in IL-$4^{-/-}$ or NKT-cell-deficient J$\alpha18^{-/-}$ hosts compared with WT hosts (Pillai et al. 2009). Furthermore, injection of WT BALB/c NKT cells markedly increased donor Treg division and accumulation in both J$\alpha18^{-/-}$ and IL-$4^{-/-}$ hosts. The ability of NKT cells to restore Treg division was dependent on their production of IL-4, because IL-$4^{-/-}$ NKT cells failed to significantly increase Treg expansion (Pillai et al. 2009). NKT cells from irradiated mice develop a Th2 bias via unknown direct or indirect mechanisms, with increased IL-4 secretion after activation (Lan et al. 2001, 2003). However, in control experiments, two other key cytokines secreted by host NKT cells, IFN-γ and IL-10, were not required for either donor Treg expansion or protection against early GVHD colitis after TLI/ATS and allogeneic BMT (Pillai et al. 2009). Cumulatively, these studies demonstrate that iNKT cells can induce expansion of Treg through an IL-4-dependent mechanism.

3.5 Protective NKT in a Model as Asthma Mediated by IL-33(IFN-γ)

In the lung inflammation model triggered with IL-33, a protective NKT subset, which expressed IFN-γ, has been studied by Bourgeois et al. (2011). IL-33, a member of the IL-1 cytokine family (Schmitz et al. 2005), has been shown to induce lung inflammation and AHR when administered repeatedly into the lungs of mice (Kondo et al. 2008). Interestingly, the inflammation, including eosinophil and neutrophil recruitment, and local production of eotaxin and keratinocyte chemoattractant chemokines, was markedly increased in IL-33-treated iNKT-cell-deficient (J$\alpha18^{-/-}$) mice (Bourgeois et al. 2011). By contrast, lung inflammation decreased after adoptive transfer of iNKT cells 1 week prior to the 7-day treatment with IL-33, restoring the WT inflammatory response in J$\alpha18^{-/-}$ mice. Moreover, IFN-γ levels were reduced in iNKT-cell-deficient J$\alpha18^{-/-}$ mice, while IL-4 levels remained unchanged. These data suggested that IL-33 induced IFN-γ production in NKT cells. The critical role of iNKT cells in reducing lung inflammation through their IFN-γ production was definitively confirmed by transferring iNKT cells from IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice into J$\alpha18^{-/-}$ mice 1 week prior to the 7-day IL-33 treatment. Unlike their transferred WT counterpart, transferred IFN-γ-deficient iNKT cells failed to reduce the recruitment of inflammatory cells in the airway passage and failed to reduce chemokines such as eotaxin and KC in the bronchoalveolar lavage fluid, supporting the conclusion that IFN-γ-producing iNKT cells counteracted IL-33-driven lung inflammation (Bourgeois et al. 2011). Although the expression of either CD4 or T-bet molecule in these subsets

was not assessed, those IFN-γ-producing iNKT cells in IL-33 model have very similar feature of suppressive ability as that of the DN NKT subset in the lungs in the influenza model (Blanden 1974).

3.6 Type II Inhibitory NKT Cell Effect on Cancer (IL-13)

While a number of studies have shown that NKT cells can augment antitumor immunity through the production of IFN-γ (Crowe et al. 2005; Smyth et al. 2000; Toura et al. 1999), Berzofsky et al. showed that NKT cells could suppress antitumor responses through the production of IL-13 (Terabe et al. 2000, 2003, 2004, 2005; Terabe and Berzofsky 2007). These investigators found that the suppressive NKT cell was a type II NKT cell that lacked the canonical invariant T-cell receptor, in contrast to type I NKT cells, which express the invariant receptor and that mediate tumor protection. Further, these two subsets of NKT cells counter-regulated each other. The IL-13 produced by the NKT cells acted on a $CD11b^{+}$ $Gr\text{-}1^{+}$, but probably $CD11c^{-}$ myeloid lineage cell through the IL-13 receptor, inducing TGF-β production, which suppressed the CTL response (Terabe et al. 2003). The pathway could be completely abrogated by removing the type II NKT cell or the myeloid cell or by blocking the IL-13 or the TGF-β (Terabe et al. 2003). Taken together, they identified an immunoregulatory circuit initiated by NKT cells producing IL-13 in response to tumor growth that induced myeloid cells to produce TGF-β that inhibited cytotoxic T-cell-mediated tumor immunosurveillance.

4 Conclusions and Perspectives

Infections have profound effects on the development of the immune system, especially the influence on asthma. Accumulated studies have revealed that certain infections can affect innate immunity, such as NKT or APCs, and can persist their effect for some time after infection, which may provide a mechanism for Hygiene Hypothesis. In a recent study, infection with H3N1 influenza A virus or treatment of an NKT-stimulating glycolipid from *H. pylori* in suckling mice protected the mice as adults against the development of OVA-induced AHR, revealing a novel suppressive DN IFN-γ-secreting NKT cell subset in mice. Moreover, other subsets of suppressive NKT cells with various characterizations have been studied in different models. The mechanism for those suppressive NKT cell subsets may be through direct or indirect interactions with Treg, macrophage, as well as pDC, or secretions of certain cytokines, such as IFN-γ, IL-4, or IL-10, to control the immune responses. However, compared to previous asthma-inducing NKT subsets which have been revealed in several asthma models, those new regulatory NKT subsets have drawn more attention to their ability of suppressing the immune responses, which have been found in ACAID, diabetes, and transplant tolerance. Taken

together, these studies bring the idea that a balance of effect and regulatory immune cells may exist in the lung to maintain the immune homeostasis; several new suppressive subsets, such as iTR35 and suppressive NKT cells, of interest, have been revealed by their importance in controlling autoimmune disease, such as asthma. Therapies that enhance the development of those regulatory subsets in patients with allergic disease and asthma may be very effective in the prevention of these disorders.

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Immune Homeostasis of the Gut

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Abstract Immune responses in the gastrointestinal tract are highly regulated to prevent inappropriate responses to the gut microbiota and food antigens while protecting the host from invasive infections. The following chapter describes the unique physiological adaptations in the intestine that allow for the appropriate induction of immune responses and the effector sites where these responses take place. Homeostasis is described as a process that involves the *compartmentalization*, *monitoring*, and *selection* of the members of the gut microbial ecosystem. The roles of the barrier and the microbiome as active participants in this process are highlighted. The role of important molecules such as retinoic acid, TGF-β, and microbiota-derived signals in directing tolerogenic responses is also discussed. Examples of disease states associated with alterations in the microbiome are used to emphasize the importance of maintaining a well-balanced gut microbial ecosystem. Together these mechanisms maintain the immune balance in the gastrointestinal tract that is essential for health.

1 Introduction

In most tissues, the sensed presence of a bacterial or viral intruder triggers a rapid immune response that, if successful, results in the clearance of the pathogen and long-term memory to a secondary challenge. The mucosal surfaces, on the other hand, are inherently nonsterile environments. In particular, the gastrointestinal (GI) tract is constantly exposed to the "outside" world. Colonization of the gut with a complex microbial ecosystem, the microbiota, is essential to the optimal fitness of the host. The most advantageous outcome of an immune response in the gut is not

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necessarily the clearance of nonself components, but rather the control and management of the microbial ecosystem. It has been argued that the development of an adaptive immune system has been driven in part by the need to support an increasingly complex microbiome (Lee and Mazmanian 2010). Indeed, even nonvertebrate species such as coral forming cnideria and squid are associated with bacterial symbionts that are carefully and precisely selected to colonize their hosts (Söderhäll et al. 2010; McFall-Ngai 1999). Therefore, it should not seem surprising that homeostasis of the immune system in the gut is reliant on signals from the microbiome. The homeostatic state between response to potentially harmful infection, maintenance of a well-balanced microbial ecosystem, and the prevention of a pathologic inflammatory response is, therefore, a joint effort on the part of the host's immune system, the physiology of the host, and the composition of the gut microbial community. Specialized structures and adaptations of the gut-associated lymphoid tissue (GALT) serve to *compartmentalize*, *monitor*, *and select* the microbial components that reside in the gut to maintain immune homeostasis.

1.1 The Immune Physiology of the Gastrointestinal Tract

Several specialized adaptations in the small and large intestine contribute to immunological homeostasis in the gut. These include the epithelial cell tight junctions that make up the barrier, a layer of mucus, the production of antimicrobial peptides, and an abundance of secretory IgA antibody (Fig. 1). The small intestine is defined, moving proximally from the stomach, as the duodenum, jejunum, and ileum. The large intestine includes the cecum (in mice) and the colon. The epithelial cells that make up the intestines include enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Of these, the enterocytes are primarily responsible for food absorption. Goblet cells produce mucus, enteroendocrine cells make hormones, and Paneth cells secrete antimicrobial peptides (Santaolalla et al. 2011). More lymphocytes reside in the GALT than in all of the secondary lymphoid organs combined. Intraepithelial lymphocytes (IEL) are found interspersed above the basement membrane of epithelial cells. The epithelial lamina propria contains the dendritic cells (DCs), antibody-secreting B cells, macrophages, and effector T cells that constitute the effector site of the GALT. Organized lymphoid structures including the Peyer's patches (PP), isolated lymphoid follicles (ILFs), and the mesenteric lymph node (MLN) form the inductive sites of the GALT. The MLN is perhaps the most important of these, draining both the small intestine and the ascending colon (Carter and Collins 1974).

PP are organized structures of B cells, T cells, and antigen presenting cells (APCs) and are found on the antimesenteric side of the small intestinal lamina propria. They resemble peripheral lymph nodes closely in both composition and structure, and include well formed germinal centers (Coles et al. 2010). However, unlike peripheral lymph nodes, PPs do not have afferent lymphatics. Instead, the luminal side of the organ includes a specialized follicle-associated epithelium

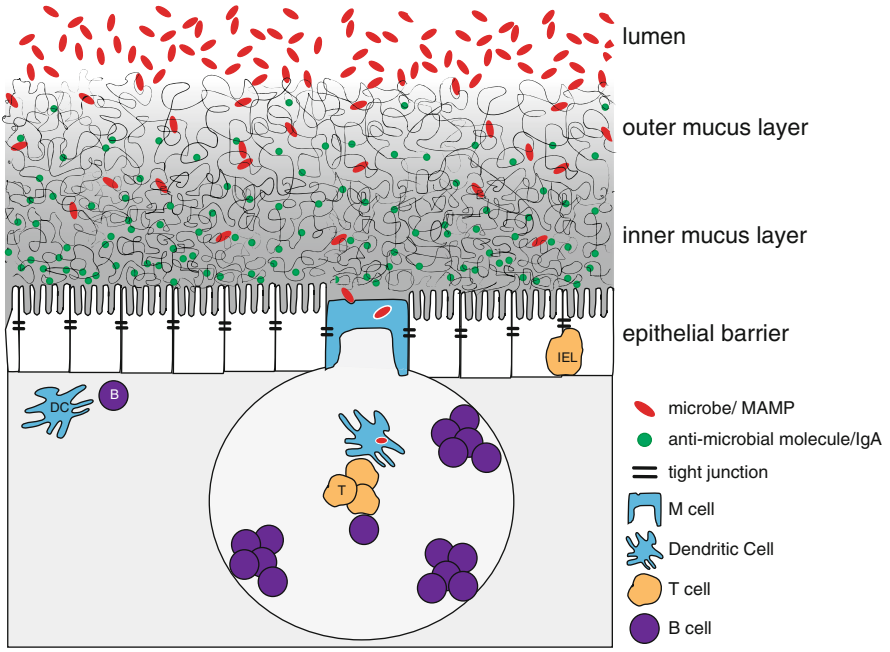

Fig. 1 Specialized adaptations of the mucosal immune system maintain homeostasis. Most microbes in the gut are not in direct contact with the epithelia and are compartmentalized by a thick layer of mucus to the lumen. Antimicrobial peptides and sIgA are secreted into this mucus to control microbial populations. Tight junctions that restrict the free passage of material to the LP connect the epithelial barrier. Interspersed between epithelial cells are IELs that can respond quickly to microbial challenge. In the LP, cells of the immune system are in place to respond to any microbes that breach the barrier or to capture antigens for the induction of oral tolerance. M cells aid active sampling of the microbiota and deliver antigens to isolated lymphoid follicles or PP where APCs stimulate T cells and B cells and induce germinal center reactions

(FAE) that contains M cells, which are named for their microfold structure. Unlike the surrounding enterocytes, M cells have short blunted villi, a poorly organized brush border, and a thin layer of glycocalyx (Corr et al. 2008). This property allows them to mediate transepithelial transport from the lumen to deliver bacteria or antigens to the immune cells located below in the PP (Owen and Jones 1974). A large invagination in the M cell facing the PP is closely associated with DCs, B cells, and $CD4^+$ T cells (Bjerke et al. 1988) (Yamanaka et al. 2001).

M cells are associated not only with PPs but also with the ILFs that are found throughout the small and large intestine. Unlike PPs, which can contain multiple B cell follicles, ILFs have only one B cell follicle but are still organized structures in which germinal center reactions and IgA synthesis takes place (Ivanov et al. 2006). The development of both ILFs and PPs requires the presence of $ROR\gamma t^+$ lymphoid tissue inducer cells (LTi). However, the development of PPs occurs during embryogenesis, while ILFs develop after birth. Furthermore, the development of ILFs requires signaling from the microbiota, as germ-free mice do not have these

structures. Although PPs are reduced in size in germ-free animals, they are still present. Reconstitution of germ-free mice with a commensal microbiota rescues ILF development (Lorenz et al. 2003).

Throughout the small and large intestine, heterogeneous IEL are interspersed between epithelial cells. These cells are usually $CD8^+$, can bear a T cell receptor of either the αβ or γδ lineage, and have diverse functions such as responding to infection, maintaining barrier integrity, regulating immune homeostasis, and controlling adaptive immune responses (Sheridan and Lefrancois 2010; Leishman et al. 2001). The majority of γδ IEL express a CD8αα co-receptor, which mediates interaction with nonclassical MHC-I molecules (Leishman et al. 2001). Many IEL produce the proinflammatory cytokines IL-17 and IFNγ and are directly cytolytic ex vivo (Martin et al. 2009; Sheridan and Lefrancois 2010). Interestingly, the lytic activity of γδ IEL is dependent on stimulation by the microbiota, as cells from germ-free mice do not have this capability (Lefrancois and Goodman 1989).

Luminal antigens are presented to naïve T cells in the MLN. DCs from the lamina propria capture antigen and travel to the MLN via afferent lymphatics to activate T cell and germinal center responses and antibody production. The MLN has been called the "firewall" of the GALT because it prevents the access of bacteria that have breached the barrier into the systemic circulation and is the primary site for the induction of tolerance to dietary antigens (Macpherson et al. 2009; Macpherson and Smith 2006). Macpherson et al. have shown that small numbers of bacteria that are normally nonpathogenic gain access to the MLN even in fully immunocompetent inbred mice. Migratory intestinal DCs selectively take up these bacteria and carry them to the MLN. These DCs are poor at killing the bacteria they carry, which allows time for an immune response to take place, but also introduces the possibility that microbes can be spread systemically. Under conditions in which the adaptive immune system and the MLN are intact, an IgA response is generated in the MLN, these bacteria are quickly controlled, and none reach the spleen. When the MLN is surgically removed, however, these same microbes rapidly transit to the spleen (Macpherson and Uhr 2004). Similarly, when soluble antigens are fed in small quantities, mucosal DCs are capable of capturing antigens and trafficking preferentially to the MLN, where they can present antigen to T cells and induce a tolerogenic immune response. This process does not take place if the MLN is surgically removed or if DCs cannot traffic from the mucosa to the MLN (Worbs et al. 2006). Although PPs are also inductive sites, their absence does not prevent the induction of oral tolerance, highlighting the importance of MLN as the primary inductive site of the GALT (Kunkel et al. 2003).

Lymphocytes stimulated in the MLN have a gut-homing phenotype and selectively traffic back to the GALT. This pattern of trafficking is mediated by the integrin β_7 in combination with α_4 and α_E (CD103) as well as the addressin CCR9. Expression of both CCR9 with $\alpha_4\beta_7$ allows for homing to the small intestine, which expresses the CCR9-ligand CCL5. Homing to the large intestine also requires the expression of $\alpha_4\beta_7$, but is less reliant on CCR9 (Villablanca et al. 2011). The high endothelial venules of the MLN as well as endothelial cells of PP and the lamina propria express MAdCAM-1, the ligand for $\alpha_4\beta_7$ (Gorfu et al. 2009).

Naïve B and T cells express low levels of $\alpha_4\beta_7$ in the circulation (Erle et al. 1994). Upon encounter with MAdCAM-1 these cells enter the MLN and are able to interact with their cognate antigen, when it is available. Interaction with mucosal DCs upregulates the expression of $\alpha_4\beta_7$ and CCR9 on these cells (Mora et al. 2003; Johansson-Lindbom et al. 2003). Retinoic acid (RA), a metabolite of vitamin A produced by MLN-derived $CD11c^+$ DCs, is responsible for this imprinting; IgA producing antibody-secreting cells and $CD25^+Foxp3^+$ T regulatory cells, as well as effector $CD4^+$ cells, can enter the small intestine when stimulated by RA producing mucosal DCs (Mora et al. 2006; Siewert et al. 2007; Hall et al. 2011a; Hall et al. 2011b). The paramount importance of RA in inducing homing to the gut is underscored by the finding that vitamin A-deficient animals show defective protection to gastrointestinal challenge after intramuscular vaccination (Kaufman et al. 2011).

The epithelial cells that make up the barrier, tight junctions of epithelial cells, mucus, PP, ILFs, and the MLN are the specialized structures and adaptations critical to the maintenance of immune homeostasis. The detailed mechanisms associated with these functions will be expanded upon below.

1.2 Compartmentalization *of Microbial Components*

A single layer of epithelial cells forms the barrier, and these cells are an essential part of maintaining homeostasis. Enterocytes separate the lamina propria from the trillions of microbes that populate the gut while allowing for food and water absorption (Whitman et al. 1998). The epithelial cells that make up the gut barrier are connected by tight junctions that restrict the passage of molecules based on size (Turner 2009). The size discrimination differs depending on the location in the intestines, with the crypts allowing larger solutes to pass, and the villi being less permeable (Fihn et al. 2000). It should be noted that the majority of the bacteria that make up the microbiome are not in direct contact with the epithelial layer. They are in fact separated by a thick layer of mucus, in humans comprised primarily of mucin Muc2, a heavily glycosylated protein (Gum et al. 1994). The mucus layer is most dense and closest to the epithelium and becomes less dense toward the lumen (Atuma et al. 2001). Mucus is constantly being generated by goblet cells at the epithelial layer, and is secreted into the lumen. It is, therefore, not surprising that the density of microbes decreases from the lumen (10^{11} colony forming units (CFU) per g) to the outer mucus layer (10^6CFU per g) (Johansson et al. 2008; Van den Abbeele et al. 2011). Close contact with the epithelial cells of the barrier seems to be limited to a specific niche of the microbiota, particularly *Lachnospiraceae* and *Ruminococcaceae*, members of the phylum *Firmicutes* (Nava and Stappenbeck 2011). The ability to lessen direct contact between the epithelium and the microbiota is essential in protecting the host from inappropriate inflammatory responses, as mice which lack colonic mucus are more susceptible to chemically-induced (DSS) colitis (Petersson et al. 2011). More importantly, embedded within

the mucus are a variety of antimicrobial components that *select* for both the types and numbers of bacteria that can reside within any given layer. These molecules are discussed in detail in Sect. 1.4.

1.3 The Immune System Constantly Monitors *the Composition of the Microbiota*

Even though many layers of protection are in place to compartmentalize the microbiota, it is important to emphasize that this does not imply that the host ignores the microbiome. Extensive mechanisms are in place to sense and *monitor* the identity, number, and location of microbes. The epithelial layer itself is able to sense bacterial products. Bacterial components can gain access to the immune system by transepithelial transport through M cells, or by pIgR-mediated transport via IgA. In the lamina propria, below the basement membrane, several subsets of DCs await any nonself components. Sensing of the microbiome is essential in maintaining homeostasis as these mechanisms initiate the appropriate innate and adaptive responses to antigens in the intestinal lumen.

1.3.1 The Importance of the Epithelia in Detecting Microbial Signals

Microbe-associated molecular patterns (MAMPs) are conserved components on microbes, regardless of pathogenic capability, that are recognized by pattern recognition receptors (PRRs) (Medzhitov and Janeway 2002). Intestinal epithelial cells (IECs) can sense bacterial products directly by detecting MAMPs with PRRs such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Systemically, recognition of a MAMP by a PRR would initiate an inflammatory response. Obviously this strategy of detection and response is not desired in an organ that normally houses trillions of bacteria, the majority of which perform functions beneficial to the host. Therefore, PRRs in IECs are polarized in their expression; for example TLR5 is expressed on the basolateral side of IEC. This means that the MAMPs sensed by TLR5 will only be detected if the microbe or microbial product has breached the barrier (Gewirtz et al. 2001). The expression of TLR2, TLR4, MD2, and CD14 has also been found to be lower on the apical side of IECs, thereby producing hyporesponsiveness to microbial antigens (Otte et al. 2004). The level of MD2, a secreted accessory molecule necessary for TLR4 signaling, is controlled in part by degradation by Paneth cell-derived trypsin (Cario et al. 2006) as well as by genetic silencing (Vamadevan et al. 2010). TLR4 is expressed at a higher level at the base of the crypts, and decreases in expression as epithelial cells mature and move closer to the lumen (Furrie et al. 2005). Therefore, a response occurs only if bacteria penetrate into the crypt epithelium. Stimulation by the microbiota has been adapted to benefit the host and a low level of tonic signaling

by TLRs is necessary for epithelial maintenance. TLR2 stimulation on intestinal epithelial cells improves tight junction integrity and protects against chemically (dextran sodium sulfate, DSS) induced colitis (Cario et al. 2004, 2007). A level of tonic TLR signaling is necessary to compartmentalize the microbiota. When a normally noninvasive bacterium is introduced into germ-free mice which lack the signaling adaptor molecules MyD88 or Trif ($MyD88^{-/-}$ $Ticam1^{-/-}$), bacterial loads in the spleen increase. The mice are smaller in size and less robust, and have compensatory serum antibody responses (Slack et al. 2009).

In the case of pathogenic bacterial invasion, it is imperative that TLRs produce an appropriate immune response, even in the GALT. Mice with a TLR4 mutation on stromal cells are more susceptible to invasive *E. coli* infection (Schilling et al. 2003). Stromal defects in the TLR-signaling adaptor MyD88 result in an inability to clear invasive *Listeria monocytogenes* (Brandl et al. 2007) or *Citrobacter rodentium* (Lebeis et al. 2007). Protection from pathogens is not restricted to the stimulation of an inflammatory adaptive immune response but also includes the initiation of barrier repair, chemoattraction of immune cells to the site of infection, and the secretion of antimicrobial peptides by IECs (Lebeis et al. 2007; Brandl et al. 2007). Although the cell surface PRRs expressed on IECs are hyporesponsive, intracellular PRRs such as the NLRs may play an important role in detecting "misplaced" bacteria or viruses, such as invasive species. NLRs are intracellular PRRs that recognize a variety of bacterial antigens as well as molecules associated with cell damage (Wells et al. 2011). NOD1 and NOD2 expression in the small intestine is associated with responsiveness to the synthetic peptidoglycans meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively (Wells et al. 2011). As with TLR expression, NOD expression is tightly controlled in the epithelium with NOD2 being confined to Paneth cells, at least in human samples (Lala et al. 2003). Human intestinal epithelial cells express NOD1 constitutively. In vitro, stimulation of NOD1 by invasive, intracellular *E. coli* results in NF-κB signaling, and blockade of this pathway prevents the upregulation of chemoattractants that would be responsible for immune cell recruitment during a bacterial infection (Kim et al. 2004).

The host and certain members of its microbiota have co-evolved to use these detection methods to shape the immune response and therefore microbial ecology (Sansonetti 2010). For example, the lipopolysaccharides (LPS) of certain microbial MAMPs from symbionts are modified to avoid detection by PRRs; the pentacylated lipid A of *Bacteroides* is less immunogenic than the hexacylated agonistic Lipid A of *Enterobacteriaceae* (Munford and Varley 2006). *Bacteroidetes* outnumber *Enterobacteriaceae* by 10^4–10^6 in healthy individuals and a shift in this balance is often associated with Inflammatory Bowel Disease (IBD) (Swidsinski et al. 2002; Peterson et al. 2008). Another example is the flagellum of *Helicobacter sp.*, which binds TLR5 poorly (Andersen-Nissen et al. 2005). However, it also causes some defects in motility. This demonstrates the selective pressure that may be in effect for bacteria to be less agonistic. Although this is one strategy by which commensals achieve tolerance, pathogens such as *Y. pestis* use a similar strategy to avoid detection and therefore achieve virulence (Montminy et al. 2006).

Together these findings demonstrate that appropriate *monitoring* of bacterial components by intestinal epithelial cells is an important part of maintaining immune homeostasis in the gut. Over-stimulation by the vast number of bacterial ligands in the lumen is prevented by the regulation of both PRR expression levels and by their cellular and subcellular localization. Invasive bacteria do not escape detection by PRRs; indeed PRR-induced innate immune signaling is an essential prerequisite for the induction of adaptive immunity.

1.3.2 Mucosal Dendritic Cells Subsets Maintain Homeostasis

DCs are professional APCs that capture antigen, migrate to a nearby draining LN, and present peptide on major histocompatibility complex (MHC) molecules to T cells. DCs that acquire antigen and microbial stimulation via PRRs at the same time activate naïve T cells into an effector phenotype. In the mucosa, this type of activation must be tightly controlled; PRR ligands are highly abundant but an aberrant inflammatory response to the microbiota is not desired. Furthermore, because the gut is the site of nutrient absorption, a variety of nonmicrobial yet nonself antigens in the form of food are also taken up by DCs. APCs in the mucosa are, therefore, also responsible for initiating oral tolerance to these antigens. However, in the case of an invasive bacterial infection, mucosal DCs must be ready to respond and generate an appropriate immune response. Therefore, it may not be surprising that many different DC subsets exist in the gut with some being more adept at mediating specific types of immune responses than others.

DCs reside throughout the lamina propria of the small and large intestine and are present in PP and ILFs. They can gain access to antigens that have been transported by M cells or IgA or have breached the barrier. They may also extend dendrites between epithelial cells to directly sample luminal antigens, although the relevance of this population in initiating immune responses is controversial since these cells have been shown to be nonmigratory (Geissmann et al. 2010; Schulz et al. 2009). Many tissue-resident DCs do regularly migrate to the MLN via the afferent lymphatics, and this migration does not depend on activation by bacterial components as germ-free, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice have equivalent numbers of migratory DCs (Wilson et al. 2007). Upon migration to the MLN, DCs will interact with T cells to produce an appropriate immune response. The type of response induced depends on the context in which antigen was acquired and the phenotype of the DC.

The myeloid-derived cells of the gut, including DCs and macrophages, are highly heterogeneous. Many studies define DCs as distinct populations based on their expression of the molecules CD11c, CD11b, DEC205, CD8α, CD103, CX_3CR1, and MHC-II and demonstrate that distinct populations of DCs stimulate different T cell responses. Although many of these markers are found on other myeloid cells, such as macrophages, and there is some disagreement on how to best characterize DCs versus macrophages, it is becoming clear that in the GALT distinct populations of APCs lead to tolerogenic or regulatory T cell responses,

while others are responsible for inflammatory T cell responses (Geissmann et al. 2010). The CD11c marker is most often associated with DCs, but within the $CD11c^+$ subset there is diverse expression of the other markers. Of particular interest are CD103 and CX_3CR1, as these cell surface markers have been suggested to define tolerogenic and proinflammatory populations, respectively.

$CD11c^+$ $CD103^+$ DCs have been shown to be the migratory population that is largely responsible for the de novo induction of $Foxp3^+$ Tregs in response to orally administered antigen (Matteoli et al. 2010; Sun et al. 2007). The role of $CD11c^+$ $CD103^+$ DCs in inducing tolerogenic responses has been the subject of extensive and ongoing research. In the setting of normal homeostasis and health, epithelial cells release TGF-β and retinoic acid (RA), which "condition" DCs in the lamina propria (Iliev et al. 2009a; Iliev et al. 2009b). Part of this conditioning includes the upregulation of RALDH, an enzyme that allows for the metabolism of dietary vitamin A into RA. Therefore, when the conditioned DC migrates to the MLN, it has the ability to itself produce RA and TGF-β, the combination of which has been shown to induce the de novo generation of $Foxp3^+$ Tregs (Sun et al. 2007), and support the trafficking of these cells into effector sites (Kaufman et al. 2011; Hall et al. 2011a; Hall et al. 2011b). These cells also express high levels of indoleamine oxidase (IDO), another molecule that supports Treg development (Matteoli et al. 2010). Constitutive β-catenin signaling has also been reported to drive the production of RALDH, IL-10, and TGF-β in lamina propria DCs (Manicassamy et al. 2010). The role of the epithelium in driving this signaling cascade is under investigation. New studies show that the role of RA producing $CD11c^+$ $CD103^+$ DCs is context dependent. In an inflammatory environment $CD11c^+$ $CD103^+$ DC promote proinflammatory IL-17 responses, and the production of RA enhances the generation of these effector T cells (DePaolo et al. 2011; Hall et al. 2011a).

$CD11c^+$ CX_3CR1^+ cells in the lamina propria have been shown to extend processes between epithelial cells and freely sample luminal antigens while maintaining epithelial tight junctions (Chieppa et al. 2006). These cells are most likely strictly tissue resident, and may not traffic to the MLN (Schulz et al. 2009). CX_3CR1^+ cells play a role in the clearance of *Salmonella*. They are recruited to the epithelium upon *Salmonella* infection in a MyD88-dependent manner (Arques et al. 2009). Mice in which one allele of CX_3CR1 is inactive have increased susceptibility to infection with *Salmonella* (Niess et al. 2005). CX_3CR1^+ cells are reduced in the colonic and small intestinal LP of germ-free mice, suggesting that their development or recruitment into the LP is dependent on signaling from the microbiota. Interestingly the number of $CD103^+$ DCs is unaltered in the LP of germ-free mice but is reduced in their MLN (Niess and Adler 2010). Ex vivo, the CX_3CR1^+ population preferentially secretes proinflammatory IL-6, IL-12p40, and IL-23 upon CpG stimulation, and induces increased numbers of IFN-γ-producing T cells, while the $CD103^+$ population preferentially secretes IL-10 and IL-22 and induces IL-10 producing T cells in culture (Niess and Adler 2010).

Recently it has been demonstrated these two cell types can work together to generate and maintain oral tolerance (Hadis et al. 2011). Hadis et al. proposed a two-step model of oral tolerance in which $Foxp3^+$ Tregs are generated in the MLN, presumably by the migratory $CD11c^+$, $CD103^+$ DC population. After undergoing

several rounds of proliferation, these Tregs were shown to migrate into the lamina propria in an ITGB7 (β7), MAdCAM-1-dependent manner. However, oral tolerance could not be maintained in the absence of CX_3CR1^+ cells, and this population was shown to be responsible for producing IL-10 and supporting additional expansion of orally induced Tregs in the LP (Hadis et al. 2011).

Therefore, the literature suggests that there is a division of labor in the LP APC populations. $CD11c^+$ $CD103^+$ cells appear to be the migratory APC population responsible for the induction of tolerogenic responses, while the $CD11c^+$ CX_3CR1^+ cells mediate responses in the local LP including inflammatory responses. This division, however, is not strict and may depend on the local context and inflammatory state of the gut.

1.4 Homeostasis Is Maintained by Selecting *the Microbial Composition*

In response to the detection of the microbial community in the gut, the host actively maintains, selects, and controls these microbes through signals derived from both the innate and adaptive immune systems. Innate signaling molecules include antibacterial defensins, cathelicidins, and C-type lectins such as RegIIIβ and RegIIIγ. These are secreted by enterocytes and, particularly, Paneth cells. These molecules are highly conserved among species and serve as a broad spectrum of protection against many classes of bacteria and fungi (Boman 1995; Zasloff 2002). They also have toxin-neutralizing activity, and roles in the chemotaxis of immune cells, wound healing, and DC maturation (Salzman 2008).

Defensins not only maintain the numbers of bacteria but can also be selective. A study by Salzman et al. found that expression of a human defensin in mice deficient in their own alpha defensin alters the composition of microbial communities, while maintaining the total number of bacteria. Notably, there was a loss of segmented filamentous bacteria (SFB) in these mice and a correlative decrease in IL-17 secreting LP T cells. (Salzman et al. 2010). These data suggest that defensins and other antimicrobial peptides have broad, generalized specificities that help to set-up/maintain the commensal microbial community composition, which, in turn, alters adaptive immunity.

The IgA antibody isotype can confer specific protection in the GALT. This antibody is the most abundant isotype in mucosal secretions and is uniquely suited to the mucosal microenvironment (Chodirker and Tomasi 1963). Secretory IgA (sIgA) in the gut is dimeric or oligomeric (Cerutti et al. 2011). Multimers of the antibody are joined by a J chain that is necessary for binding to the polymeric Ig receptor that transports IgA across epithelial cells, into the lumen (Mostov and Deitcher 1986; Brandtzaeg and Prydz 1984). Part of this receptor, then, is retained by the antibody as secretory component and prevents the degradation of the antibody in the harsh gut environment (Cerutti et al. 2011).

IgA is absent in neonates and germ-free animals and is induced upon bacterial colonization (Benveniste et al. 1971; Moreau et al. 1978; Macpherson et al. 2007).

Both T dependent and T independent pathways exist for generating IgA (Fagarasan et al. 2011). T cell-dependent IgA responses generate somatically hypermutated, affinity-matured antibody after a germinal center reaction. This type of B cell response occurs in the PP, the small intestine, the MLN, or ILFs. Binding of antigen by B cell receptors (BCR) as well as the interaction of CD40 on B cells with its ligand on T helper cells induces activation-induced (cytodine) deaminase (AID) and initiates somatic hypermutation and class switch recombination. TGF-β, which is abundant in the GALT, is an essential factor mediating class switching to IgA versus other isotypes (Kim and Kagnoff 1990). Mice with a deficiency in a TGF-β receptor on B cells have a significant impairment in serum IgA titers (Cazac and Roes 2000). Interestingly, the innate-like, peritoneal residing, B1 B cell population in these mice is increased, suggesting that these cells can compensate for IgA deficiency (Cazac and Roes 2000). T cells also support B cell survival and class switching by secreting the molecule APRIL (Macpherson et al. 2007). While in the follicle of the PP, stromal cells called follicular dendritic cells promote IgA by secreting BAFF and TGF-β after stimulation from TLRs and retinoic acid (Suzuki et al. 2010). After class switching to IgA in the PP or MLN, plasma secreting B cells can populate the LP (Macpherson et al. 2007).

T independent IgA responses occur outside of PP, in the LP, without the formation of germinal centers. Direct stimulation of B cell TLRs can replace the CD40/CD40L interaction to initiate class switch recombination. A second cytokine signal is needed to direct class switching to IgA. Epithelial cells stimulated with TLR ligands secrete APRIL and, together with IL-10 produced by DCs can mediate B cell class switch to IgA (He et al. 2007). Retinoic acid is also an important mediator of T-independent IgA production in the gut. In vitro it has been shown that mucosal DCs, IL-6, IL-5, and RA synergize to induce IgA production in a T cell independent manner (Mora et al. 2006).

Functionally, the role of sIgA in maintaining gut homeostasis has been difficult to precisely delineate because of the compensatory redundancy of the GALT. Mice deficient in the pIgR that transports IgA into the lumen are robust but have an increase in serum levels of IgG and IgA as well as signs of increased barrier permeability (Johansen et al. 1999; Shimada et al. 1999). IgA has also been shown to limit penetration of normally noninvasive bacteria into systemic organs that would otherwise cause an inflammatory response (Macpherson and Uhr 2004). In the lumen, secretory IgA has the ability to neutralize bacterial toxins, such as cholera toxin (Lycke et al. 1987). Highly glycosylated sIgA bound to bacteria may increase bacterial trapping in the mucosal matrix and prevent further invasion (Phalipon and Corthesy 2003). If microbes penetrate the barrier, IgA in the lamina propria can bind and then export the bacteria back into the lumen through epithelial cells via the pIgR (Robinson et al. 2001). A similar mechanism in M cells allows IgA-bound microbes to be transported from the lumen into the PP, increasing microbial sampling (Rey et al. 2004; Corthesy 2007). High titers of sIgA also correlate with increased protection against rotavirus infection (Feng et al. 1994). Together these data support IgA as one of the mucosal mechanisms that *compartmentalize* the microbiota.

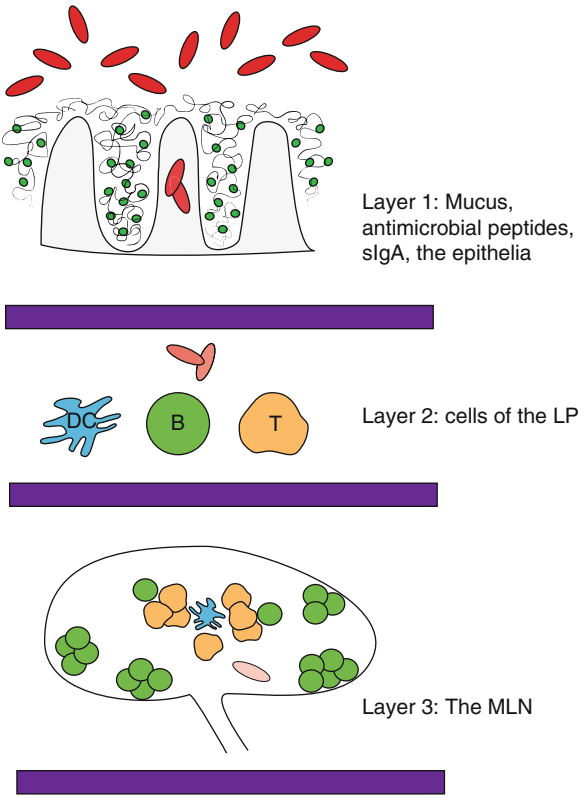

Fig. 2 Layers of protection in the mucosal immune system maintain and control the complex microbiota. At each layer redundant mechanisms are in place to compartmentalize, monitor, and control for the presence of microbial components and damage. The first layer of protection consists of mechanisms that maintain the microbiome outside of the LP, this consists of mucus, antimicrobial peptides, sIgA, and the epithelial barrier. The second layer, comprised of the cells of the LP, is in play should a microbe breach the epithelial barrier. The final layer of protection to prevent microbes from reaching systemic circulation is the MLN

Each of the mechanisms mentioned above, *compartmentalization*, *monitoring*, and *selection*, takes place at multiple sites throughout the mucosal immune system. Therefore, at each site, an invading microbe is faced with a new layer of protection (Fig. 2). The first layer of protection consists of the mucus, antimicrobial peptides, sIgA, and the epithelial cells that make up the barrier. Should a microbe pass this first layer of protection, the cells of the LP await to capture it. If these mechanisms should fail, the MLN provides a third layer of protection before a microbe reaches systemic circulation. The redundancy of the mucosal immune system reflects the challenge of maintaining and controlling the complex microbial ecosystem that is the microbiota while simultaneously providing protection from pathogenic invaders.

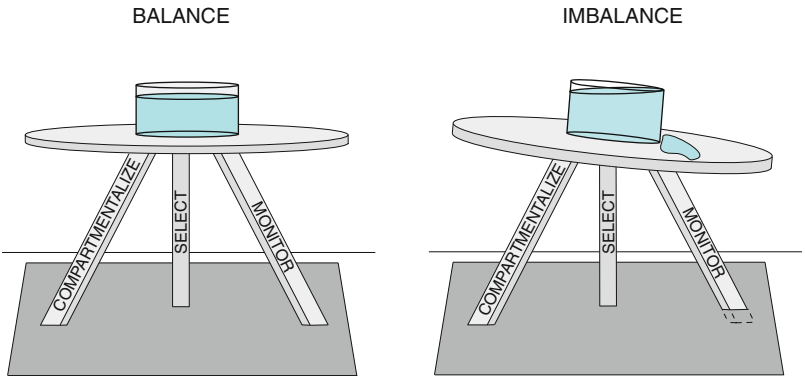

Fig. 3 The homeostasis of the microbiota is maintained by three strategies: compartmentalization, monitoring, and selection. When one of more of these mechanisms is not in place, alterations in the composition of the microbial gut ecosystem can occur. These imbalances can contribute to, drive, or initiate disease states

1.5 Impacts on Health When the Microbiota Is Not Appropriately Compartmentalized, Monitored, *or* Selected

Discussed above were the mechanisms by which the gut microbiota is *compartmentalized*, *monitored*, and *selected* and by which homeostasis is achieved. When one or more of these mechanisms fails, or if the environment is altered, homeostasis shifts, resulting in alterations to the microbiome. These alterations can result, contribute to, or drive disease states (Fig. 3). Below are described the approaches by which the microbiota is evaluated, and several examples of disease states in which alterations of the microbiota play a role.

The majority of the species that comprise the microbiome are nonculturable, and it is only recently that the increased availability and use of high-throughput sequencing and metagenomic techniques have allowed for a more accurate description of the composition of the microbiota. Germ-free and gnotobiotic (known biome) animals have also been instrumental in elucidating the importance of the microbiome in immune development and heath. It is becoming increasingly clear that a "well-balanced" microbiome is associated with proper immune function and health while dysbiosis is associated with inflammatory, allergic, autoimmune, and metabolic disease (Frank et al. 2011). However, it is not yet clear what constitutes a "well-balanced" microbiome and what factors lead to dysbiosis; the directionality of alterations to the microbiome and disease are difficult to establish in complex human immune-mediated diseases. Animal models are yielding promising results in bridging this gap. Current evidence from both human and animal models is demonstrating that diet and antibiotic use contribute significantly

to alterations in the gut microbial ecosystem and diseases associated with such changes.

The advent of relatively inexpensive high throughput sequencing has allowed for a more complete characterization of the mammalian bacterial microbiome. The bacterial 16S ribosomal RNA gene is particularly useful for surveying the composition of the microbiome. This gene contains highly conserved regions, allowing for universal amplification, and highly variable regions, which can be used to identify specific species. Therefore, information on the composition of the microbiome of an individual host can be gleaned from fecal samples, biopsies, or in the case of animal models, mucosal scrapings of particular portions of the GI tract. The limitations of current techniques do not yet allow for three-dimensional reconstruction of microbial community composition in relation to the epithelial/luminal axis. New techniques are being developed to address bacterial function at a high-throughput level as well. Until such analysis is readily available, discovery of the functional importance of particular members of the microbiota is reliant on gnotobiotic animal models. An ingenious approach to study to the direct functional effects of a complex human microbiome on health has been developed by the Gordon lab. This group has demonstrated that it can faithfully reproduce the composition of a human microbiome in germ-free mice by fecal transfer (Faith et al. 2011).

Diet has been shown to be a major driver of the composition of the microbiome. Analysis across species has shown that diet has more influence on the gut microbial ecosystem than evolutionary relationships between hosts. However, overlapping functions of members of the microbial ecosystem are associated with particular diets, suggesting that the composition of the microbiome, as selected by diet, is driven on the basis of biochemical function and not phylogeny (Muegge et al. 2011). Dietary metabolites produced by the microbiome have significant impacts on the host immune system. Mammals are incapable of metabolizing complex plant fibers, which are broken down into short chain fatty acids (SCFA) by commensal bacteria (Qin et al. 2010). The amount of fiber in the diet alters the microbial composition and the levels of these SCFA (De Filippo et al. 2010). These SCFA, in particular butyrate, have been found to play a role in gut homeostasis and intestinal health. For example, butyrate has been shown to increase the production of Muc2, the main component of mucus, in colonic cell lines, and to limit the proliferation of colon cancer lines in vitro (Hatayama et al. 2007; Willemsen et al. 2003). It has also been shown to increase epithelial cell integrity, by increasing tight junction assembly, at least at low concentrations (Mariadason et al. 1997; Peng et al. 2009). At high concentrations, butyrate may be toxic and induce increased epithelial permeability (Peng et al. 2007). The quantity and composition of SCFA produced by the microbiota could be one signal that contributes to intestinal health; dysbiosis may be associated with an imbalance or absence of SCFAs that leads to disease (Topping and Clifton 2001). However, butyrate-production is not confined to one phylogenetic group illustrating the difficulty in discerning how changes in the microbiota at the phylogenetic level result in functional alterations in homeostasis (Canani et al. 2011).

Significant effort has been put forth to characterize the effects of particular prototypical members of the microbiota on the immune system. Perhaps the most well-known example is that of segmented filamentous bacteria (SFB); the presence of this member of the order *Clostridiales* in the small intestine has been linked to the development of proinflammatory Th17 cells and robust immune responses (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). More recently, it has been shown that other, poorly defined, *Clostridia* sp. induce the generation of $Foxp3^+$ Tregs in the colonic LP of mono-associated animals (Atarashi et al. 2011). Monocolonization of germ-free animals with *Bacteroides fragilis* also induces the development of Tregs (Round and Mazmanian 2010). The "pathobiont" *Helicobacter hepaticus* seems to directly downmodulate the host inflammatory response in intestinal epithelial cells by using a Type VI-secretion system to direct the expression of anti-inflammatory molecules. Although this type of secretion system is often associated with increased pathogenicity, mutation of this secretion system results in increased colonization and inflammation in the case of *H. hepaticus* (Chow and Mazmanian 2010). The common mechanisms, if there are any, by which a specific bacterial species induces a particular T cell response are not yet clear. In the case of *B. fragilis*, it is known that polysaccharide A, a cell wall component, is sufficient to induce Tregs, implying that the MAMPs of some commensals are uniquely co-evolved to elicit an anti-inflammatory response (Round and Mazmanian 2010). In the case of SFB, this bacterium (or group of bacteria) is found to be tightly associated with PP, which may elicit signaling associated with invasion, and, therefore, a proinflammatory response.

Even if the common mechanisms by which members of the microbiota elicit particular immunological developmental changes are not yet clear, increasing evidence shows that "dysbiosis" of the commensal microbiota is associated with disease (Frank et al. 2011). One measure of the health of any ecosystem, including the gut microbiome, is the diversity of species within that ecosystem. For example, in humans, decreased diversity of the microbiome has been associated with Crohn's disease (Manichanh et al. 2006; Man et al. 2011). This inflammatory bowel disease is also associated with genetic mutations in NOD2, an intracellular bacterial PRR, (Hugot et al. 2001; Ogura et al. 2001), as well as defects in autophagy, a process involved in the degradation of both bacteria and cellular organelles (Hampe et al. 2007; Rioux et al. 2007). Together these data suggest that the composition, control, and detection of the microbiome could be drivers of Crohn's disease. However, it has been difficult to pinpoint a specific bacterial species or group that would be the causative agent of disease (Man et al. 2011).

Microbial dysbiosis is increasingly implicated in many diseases increasing in prevalence in the developed world. For example, in a mouse model, treatment with antibiotics increases susceptibility to an allergic response to food (Bashir et al. 2004). Germ-free mice that receive a fecal transfer of microbiota from obese mice gain more weight than those that received a "lean microbiome"(Turnbaugh et al. 2008). Understanding the complex interaction between environment (especially diet) and its influence on microbial function will yield a better understanding of the etiology of these diseases.

2 Summary

Immune homeostasis in the gastrointestinal tract is a complex association in which the microbial gut ecosystem, the immune physiology of the host, and the environment constantly influence each other. This type of dialectic relationship is sometimes difficult to tease apart, as each component influences all of the others, forming an altered whole that has significant impact on the overall health of the host. Unique adaptations in the host exist to *compartmentalize*, *monitor*, and *select* the microbial ecosystem of the gut. In turn the microbiota contributes to the maintenance and development of the host, and the host immune system. The environment, especially diet, has a significant impact on the selection of functional members of the microbiome as well as the overall health of the host. The homeostatic state between response to potentially harmful infection, maintenance of a well-balanced microbial ecosystem, and the prevention of a harmful inflammatory response is, therefore, a joint effort on the part of the host's immune system, the physiology of the host, the members of the gut microbial community, and is heavily influenced by the environment.

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Index

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