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Toll-like Receptors and the Eye

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The immune response to microbial pathogens relies on both innate and adaptive components.¹ The innate or immediate response is mediated in large measure by leukocytes of the blood, such as neutrophils (PMNs) and macrophages, cells that phagocytose and kill the pathogens and that concurrently coordinate additional host responses by synthesis of a wide range of inflammatory mediators and cytokines.² A primary challenge to the innate immune system is the ability to discriminate among a large number of potential pathogens from self, with the use of a restricted number of receptors. This discrimination is achieved by the evolution of a variety of receptors that recognize conserved motifs on pathogens called pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs), perhaps the best-characterized class of pattern-recognition receptors (PRRs)^{3,4} in mammalian species, play an important role in the recognition of components of pathogens and activation of innate immunity, which then leads to development of adaptive immune responses.⁵

In the eye, as in other parts of the body, the early response against invading pathogens is provided by innate immunity. It is now well established that the recognition of pathogens by the innate defense system is mediated through germ line-encoded PRRs,^{6,7} most notably, TLRs.^{4,8,9} A growing number of studies have shown that TLRs are expressed by a variety of tissues and cells of the eye and play an important role in ocular protection and defense against microbial infection.^{10–20} The TLR family of receptors links the extracellular compartment where contact and recognition of PAMPs occur and the intra-cellular compartment, where signaling cascades leading to cellular responses are initiated. Their extracellular domain contains leucine-rich repeats (LRRs), whereas the cytoplasmic domain shows a striking homology with that of the interleukin-1 receptor (IL-1R) and is referred to as the Toll/IL-1R (TIR) domain. The Toll/IL-1Rs play a critical role in host defense and inflammation.²¹ Ten TLRs have been identified in humans, whereas 13 can be found in the mouse genome.²² Individual TLRs recognize distinct microbial components or PAMPs.⁴ Recognition of PAMPs by TLRs induces the production of signals responsible for the activation of genes that are essential for an effective host defense, especially proinflammatory cytokine genes.^{8,23}

One of the first mammalian receptors identified was TLR4.^{24,25} Figure 1 shows TLRs, their known ligands (or PAMPs), downstream signaling pathways, and their role in mediating innate responses in cells. TLR4 is the extensively studied PRR and has been shown to be capable of sensing lipopolysaccharide (LPS) and initiating responses.²⁶ TLR2 has been shown to recognize a broad range of microbial components including bacterial and mycoplasma lipoproteins and yeast carbohydrates.²⁷ TLR3 is necessary for the response to double-stranded RNA (dsRNA),²⁸ TLR5 for the response to bacterial flagellin,²⁹ TLR7 and -8 for the response

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to single-stranded RNA (ssRNA),^{30,31} and TLR9 for the response to the unmethylated CpG motifs found in both bacterial and viral DNA.^{32,33} There are no specific ligands identified for TLR1 and -6; however, they are known to form heterodimers with TLR2, dictating the specificity of TLR2 ligand recognition.³⁴ Little is known about TLR10.³⁵

Toll-like Receptors and Downstream Signal-Transduction Pathways

Recognition of PAMPs by TLRs results in the activation of signaling events that induce the expression of cytokines, chemokines, and costimulatory molecules that control the activation of antigen-specific and nonspecific adaptive immune responses.^{36,37} Different TLR ligands exert distinct, but sometimes overlapping, biological effects that can be attributed to the activation of unique and common signaling pathways leading to the induction of unique and common sets of genes.⁸

MyD88-Dependent Pathway and Proinflammatory Cytokine Production

On ligand-binding, most TLRs recruit the adapter molecule MyD88 (myeloid differentiation protein 88) through homotypic interactions with a TIR domain present in its C terminus.^{8, 38,39} MyD88 consists of an amino (N)-terminal death domain (DD) and a carboxyl (C)-terminal TIR domain separated by a short linker sequence. MyD88 is recruited to TLRs through TIR-domain–TIR-domain interaction. MyD88, in turn, recruits IL-1R-associated kinase (IRAK) and IRAK-4 to the TLR complex through a DD–DD interaction. IRAKs contain an N-terminal DD and a central serine/threonine-kinase domain.⁴⁰ The binding of MyD88 to IRAK4 and IRAK results in their phosphorylation and activation.^{38,39} Phosphorylated IRAK then dissociates from MyD88 and becomes associated with TRAF6 (tumor necrosis factor receptor-associated factor 6).⁴¹ TAK-1 (TGF- β -activated protein kinase), constitutively associated with TAK-1-binding proteins, TAB-1 and -2, then associates with the TRAF6-IRAK complex, whereby TRAF6 in a ubiquitination-dependent manner triggers the phosphorylation and activation of TAK1.⁴¹ TAK1 can then activate kinase cascades, leading to the activation of MKKs, and the I κ B kinase complex, and ultimately to the activation of p38, JNK, and NF- κ B, respectively.^{8,42–45} Activation of these pathways is essential for the expression of multiple genes that include cytokines and chemokines such as TNF- α , IL-6, IL-8, IL-18, and MIP-1, as well as adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and E-selectin.⁴⁶

MyD88-Independent Pathway and Interferon Production

TLR3 and -4 are unique members of the TLR family in that they signal through an MyD88-independent pathway, leading to activation of the transcription factors IRF (interferon regulatory factor)-3 and -7.⁴⁷ This in turn leads to the production of IFN- β , inducible nitric oxide synthase, and the chemokines RANTES and interferon inducible protein (IP)-10.⁴⁸ This pathway has been shown to be mediated by TRIF (the TIR domain-containing, adapter-inducing IFN- β protein).⁴⁹

While the activation of NF- κ B and MAPK in response to TLR2, -5, -7, -8, and -9 ligands is abolished in MyD88-deficient cells, the TLR4 ligand LPS stimulation activates NF- κ B and MAPK in MyD88-deficient cells in a transiently delayed fashion.⁵⁰ Furthermore, LPS- or poly (I:C)-stimulated MyD88-deficient cells remain intact in their capacity to induce IFN-inducible genes, such as IP-10.^{28,51–53} These observations indicate the existence of a MyD88-independent pathway associated with the induction of IFN-inducible genes and with slow activation of NF- κ B. Recently, several adapter molecules sharing homology with MyD88 have been identified, including MyD88 adapter-like (Mal, also termed TIR domain-containing adapter protein),⁵⁴ TRIF,^{55,56} and TRAM (TRIF-related adapter molecule).^{54,57} MyD88 and Mal have been suggested to be involved in the early phase of NF- κ B activation in response

to LPS, whereas TRIF and TRAM are responsible for the later phase of NF- κ B activation based on studies in mice deficient in these molecules.⁵⁷ TRIF has been shown to be unique among these adapters in its ability to activate IRF3 and -7,^{54,57} leading to the induction of a distinct set of genes that include IFN- β , IP-10, and RANTES.⁵⁷ TLR3 has been shown to be activated by dsRNA associated with viral infection,²⁸ leading to interferon (IFN)- β expression.⁵⁸ Similar to TLR3, which induces type-1 IFN production, TLR7, -8, and -9 also induce antiviral responses by producing interferon (IFN)- α through the formation of a complex consisting of MyD88, TRAF6, and IRF7 as well as TRAF6-dependent ubiquitination.⁵⁹

TLRs and Their Ligands

TLRs have different expression patterns; some are located at the cell surface and others intracellularly. The expression pattern of TLRs appears to be related to the nature of their ligands. For example, TLRs, including TLR1, -2, -4, -5, and -6, which recognize bacterial and other microbial cell wall components, are distributed at the cell surface, whereas TLR3, -7, -8, and -9 which recognize nucleotide components of bacteria and viruses are found intracellularly.²² Thus, TLRs may be classified based on their ability to signal from cell membrane or intracellular compartments.

Plasma Membrane–Associated TLRs

TLR4 and Its Coreceptors—TLR4 is expressed in a variety of cell types, mostly in the cells of the immune system, including macrophages and dendritic cells (DCs). Recognition of LPS by TLR4 is complex and requires several coreceptors.⁶⁰ TLR4, MD2, and CD14 form a molecular complex that binds LPS and dramatically augments LPS responses.⁶¹ In DC and macrophages, which enjoy the relatively sterile environment of the peripheral lymphoid tissues where they are situated, TLR4 is at the cell surface and when the cells encounter LPS, transmit a signal rapidly for the activation and initiation of immune responses,^{29,62–64} thereby allowing the cells to sense PAMPs readily when encountering them. Epithelia, however, are in a unique position. They are in constant contact with bacteria (pathogenic and commensal) and bacterial products,⁶⁵ and yet, to mount an inflammatory response to them on each encounter, would be detrimental to the host. As LPS is a major virulence factor of Gram-negative bacteria, it should be beneficial for epithelia to be unresponsiveness to LPS. Several mechanisms have been reported for different epithelial cells to avoid unnecessary proinflammatory reactions to LPS exposure. These include expressing extremely low levels of TLR4 and no MD-2, a critical coreceptor of TLR4, in intestinal epithelial cells^{66,67} and intracellular localization of TLR4 in human pulmonary,⁶⁸ intestinal,^{69,70} and corneal epithelial cells.¹⁶ A recent paper, however, showed that in corneal epithelial cells, TLR4 requires LPS-binding protein and CD14 secreted in tears for its activity.⁷¹ Different human intestinal epithelial cell lines have been shown to have all three types of LPS responsiveness and TLR4 expression: (1) relative hyporesponsiveness to LPS with low level of TLR4, (2) hyporesponsiveness to LPS with intracellular TLR4 localization, and (3) highly LPS-responsive with surface expression of TLR4, suggesting that these cells may comprise different subpopulations with distinct roles in innate immune responses.⁷² TLR4 has also been shown to be involved in sensing various viruses including respiratory syncytial virus,⁷³ mouse mammary tumor virus, murine leukemia virus,⁷⁴ and Coxsackievirus B4,⁷⁵ via different viral proteins.

TLR2, TLR6, and TLR1—TLR2 recognizes a large variety of microbial products including lipoteichoic acid (LTA) from Gram-positive bacteria,^{23,62} lipoproteins from both Gram-negative bacteria and Gram-positive bacteria, mycobacteria, and spirochetes,⁷⁶ lipoarabinomannan from mycobacteria,⁷⁷ and zymosan from fungi.⁷⁸ The mechanism by which TLR2 recognizes a variety of bacterial components can be partly explained by the

association of TLR2 with TLR1 and TLR6. TLR2 forms heterodimers with TLR1 or TLR6 to determine its specificity of ligand binding. For example, TLR2/6⁷⁹ heterodimers recognize LTA^{79,80} and modulin from *Staphylococcus epidermidis*.^{81,82} Further, TLR2/1 heterodimers recognize triacyl lipoprotein/peptides of bacterial cell walls,⁸³ whereas TLR1/6 is responsible for recognition of diacyl lipopeptides from mycoplasma.⁸⁴ Thus, these TLRs seem to associate with other TLR2 to discriminate between subtle differences in PAMPs. The relative absence of TLR2 protein expression⁸⁵ and intracellular localization¹⁶ of TLR2 have also been proposed as potential mechanisms for epithelial unresponsiveness to its ligands in intestinal and corneal epithelial cells, respectively. Like TLR4, TLR2 also participates in sensing viral infection, including that caused by cytomegalovirus,⁸⁶ measles virus,⁸⁷ and herpes simplex virus-1.⁸⁸

TLR5—Flagellin is a monomeric constituent of bacterial flagella. It is a polymeric rodlike structure extending from the outer membrane of bacteria and possesses immunostimulatory properties.⁸⁹ Studies by Aderem et al.²⁹ demonstrated that flagellated, but not nonflagellated, bacteria activated TLR5, indicating that flagellin is a specific ligand for TLR5.²⁹ A stop codon polymorphism in the flagellin-binding domain of TLR5 is associated with susceptibility to legionnaires' disease,⁹⁰ highlighting the importance of TLR5 in microbial recognition, particularly at a mucosal surface. Indeed, in vitro studies revealed that TLR5 is a major sensor of epithelial cells to detect Gram-negative bacteria and to activate key signaling pathways leading to NF- κ B and proinflammatory gene activation in intestinal and corneal epithelial cells.^{12,91}

Intracellular TLRs

TLR3—Among the mammalian TLRs, four (3, 7, 8, and 9) recognize nucleic acids and are generally believed to be expressed on endosomal membranes, rather than the plasma membrane of cells; hence ligand-binding by these TLRs occurs in the lumen of the intracellular vesicles.⁹² It has been suggested that nucleic acids from bacteria or viruses, multiplying within a cell, can be captured in membranous vesicles and brought to the TLRs in the endosomes. Alternatively, extracellular nucleic acids released from damaged tissues or cells, infected or uninfected, are endocytosed and presented to the internal TLRs.⁴⁷

TLR3 recognizes double-stranded (ds) RNA, a byproduct of replication of some viruses. TLR3 is unique in that it functions independently of MyD88 and requires only the adaptor TRIF to transmit signals to the nucleus. TLR3 has been implicated in infection by mouse cytomegalovirus (MCMV), *Reovirus*, and influenza virus.^{47,56,93} TLR3-dependent inflammatory responses to West Nile virus infection have been shown to be required for efficient viral entry into the brain and consequent neuronal injury, suggesting that TLRs may contribute, not only to host defense, but also to pathogenesis.⁹⁴ Of interest, Ueta et al.¹⁵ recently reported that TLR3 is expressed on the surface of human corneal epithelial cells (HCECs), and its expression is amplified by poly(I:C), a TLR3 ligand that mimics the effects of viral double-stranded RNA. Stimulation of cultured HCECs with poly(I:C) elicited the production of IL-6, IL-8, and IFN- β .¹⁵ To date, the role of TLR3 in mediating a host response to viral infection remains controversial.⁴⁷

TLR9—TLR9 recognizes DNA containing unmethylated CpG motifs common to both bacterial and viral genomes.³² The CpG motifs in bacterial DNA are unmethylated and occur more frequently.⁹⁵ Mammalian DNA, on the other hand, has a low frequency of CpG dinucleotides, and these are mostly methylated; therefore, mammalian DNA does not have immunostimulatory activity.³² CpG-DNA induces a strong T-helper (Th)-1 inflammatory response. Moreover, TLR9 shows a restricted cellular and subcellular pattern of expression. In contrast to other TLR agonists, CpG-DNA is superior in activation of DC and induction of

costimulatory molecules (e.g., CD80, CD86) and cytokines such as interleukin (IL)-12 and IL-18. This qualifies CpG-DNA as a Th1-promoting adjuvant. During infection, recognition of CpG-DNA of intracellular pathogens skews and fine-tunes the ongoing immune response and induces a long-lasting T helper 1 (Th1) milieu. In the eye of B6 mice, the result of such a Th1-mediated response to *Pseudomonas aeruginosa* infection is devastating and results in corneal perforation, whereas a T helper (Th)-2-predominant response, as in BALB/c mice, results in healing.^{96,97} An important role for TLR9 in *P. aeruginosa* keratitis has been shown in B6 mice and the efficacy of silencing TLR9 by using small-interfering (si)RNA technology to modulate disease correlated with reduced proinflammatory cytokine production, but increased bacterial load in the cornea.⁹⁸

TLR9 can also be activated by CpG DNA of HSV-1, HSV-2, and MCMV. Recognition of MCMV by DC occurs through TLR9 causing cytokine secretion and viral clearance by natural killer (NK) cells.⁹⁹ Induction of IFN- α in DC and other cells by HSV-1 is mediated by both TLR9 dependent and independent pathways.¹⁰⁰

TLR7 and TLR8—Mouse TLR7 or human TLR8 recognizes viral ssRNAs.¹⁰¹ Unlike other TLRs such as TLR3, -4, and -9, TLR7 expression is restricted to the interferon-producing plasmacytoid DC subset in humans and is induced in macrophages on viral infection, furthering the implication of TLR7 expression in antiviral responses.¹⁰² Recently, Heil et al.³⁰ and Diebold et al.³¹ demonstrated that TLR7 and -8 are required for the recognition of the single-stranded RNAs (ssRNAs) found in many viruses, leading to IFN- α production in virus-infected macrophages and DC. Furthermore, TLR7 recognizes the ss-RNA viruses in vivo and mice deficient in TLR7 have reduced responses to in vivo infection with vesicular stomatitis virus (VSV).¹⁰³

Negative Regulators

The inflammatory cytokines produced as a result of TLR signaling initiate an innate response to rid the cornea of invaders. However, if the production of proinflammatory cytokines is left unchecked, excessive cytokines can lead to severe inflammatory disease and scar formation in the cornea. Very little is known about how TLR pathways are negatively regulated.¹⁰⁴ Molecules suggested to be negative regulators of TLR-signaling pathways include MyD88 short,¹⁰⁵ SIGIRR (single immunoglobulin IL-1R-related molecule),¹⁰⁴ Tollip (suppressing IRAK),¹⁰⁶ and ST2, which sequesters the adaptors MyD88 and Mal.¹⁰⁷ Among them, ST2 and SIGIRR are members of the TLR/IL-1R superfamily that negatively regulate TLR activation and are discussed in the following sections.

ST2—ST2/T1, a member of the TLR/IL-1R superfamily, is expressed on the surface of fibroblasts, mast cells, and Th2, but not Th1 effector cells. It exists in a transmembrane (ST2L) and a soluble form (ST2).¹⁰⁸ The soluble form is induced on stimulation of fibroblasts and possibly mast cells, whereas the transmembrane form is expressed in hematopoietic tissues and in the lung.¹⁰⁹ Although ST2L has immunoregulatory properties, its ligand, cellular targets, and mode of action remain unknown. Nonetheless, administration of soluble ST2 in vivo after LPS challenge significantly reduced LPS-mediated mortality and serum levels of proinflammatory cytokines IL-6, IL-12, and TNF- α , but did not alter IL-10 or nitric oxide (NO) production.¹¹⁰ These results indicate that soluble ST2 has anti-inflammatory properties that functionally suppress a Th1-type T-cell immune response. In a lung mucosal infection model, anti-ST2 mAb inhibited eosinophil infiltration, IL-5 secretion, and IgE production,¹¹¹ suggesting that ST2 is a crucial cell surface receptor that participates in regulation of Th2 type immune responses.¹¹² Its role in microbial keratitis has not yet been explored.

SIGIRR—SIGIRR has the typical conserved TIR domain that characterizes the IL-1R and TLR superfamily, but it is structurally and functionally distinct from both.¹¹³ SIGIRR has only one immunoglobulin (Ig) domain in its extracellular portion, whereas the IL-1R family contains three Ig folds, and the TLR family shows leucine rich repeats.¹⁰⁴ Although most members of the TLR/IL-1R superfamily can activate the transcription factor NF- κ B and/or AP-1 through the MyD88-IRAK4/1-TRAF6 signaling pathway, mice endogenously lacking SIGIRR are hyperresponsive to both LPS challenge and injection of IL-1 β . Furthermore, primary kidney cells and splenocytes from SIGIRR-deficient mice show enhanced activation in response to TLR/IL-1R ligands.¹⁰⁴ Nothing is known about the functions of SIGIRR in bacterial keratitis, although preliminary evidence (Hazlett LD, unpublished data, 2005) suggests that if it is reduced by antibody neutralization, *P. aeruginosa*-induced keratitis is worsened in normally resistant BALB/c mice, and the corneas perforate.

TLRs, Leukocytes, and Adaptive Immunity

The immune system in vertebrates consists of innate and acquired arms, both of which work cooperatively to protect the host from microbial infection. Innate immunity provides the first line of host defense against bacterial growth and spread in the early phase of infection. The major players of innate immune responses are immune cells such as PMN M Φ and DCs.¹¹⁴ Recent studies demonstrate that innate immunity provides for recognition of conserved pathogen associated molecular patterns (PAMPs) through TLR/IL-1R expressed on the cell surface of immune cells.² Recognition of invading pathogens then triggers cytokine production and upregulation of co-stimulatory molecules in phagocytes, leading to subsequent activation of T cells. Thus, innate immunity is closely linked to acquired immunity that is characterized by specificity and memory, and exerted by both T and B lymphocytes. In an immune response, PMNs are the first cells to arrive at the site of infection. In addition to these cells, the acute inflammatory cell infiltrate is composed of innate immune cells such as monocytes, basophils, eosinophils and natural killer (NK) cells. Both PMN and NK cells are critical effector cells that protect the host by killing pathogenic microorganisms and infected cells. Other cells such as DC reside in most tissues and organs in the immature state, and play a seminal role in detecting invading pathogens. Classically, immature DC capture antigen by endocytic and phagocytic activity and then migrate to regional lymph nodes where they develop into mature cells with powerful antigen presenting capability, enabling them to activate antigen specific naïve lymphocytes.¹¹⁵

The importance of innate immune responses to microbial infection can be stressed by the fact that such a response is sufficient to protect the vast majority of existing animal species. Vertebrate hosts, however, have evolved an additional, more sophisticated form of defense system, adaptive immunity that possesses properties of clonal expansion and memory.³⁶ Work in recent years has also shown an essential role for Toll-like receptors (TLRs) in the activation of adaptive immunity. It is now clear that activation of DC by TLR ligands is necessary for their maturation and consequent ability to initiate adaptive immune responses. Studies of DC subsets isolated from humans and mice have shown that TLRs have distinct expression patterns. In murine species, all splenic DC subsets express TLR1, -2, -4, -6, -8, and -9,¹¹⁶ but some DC subsets (plasmacytoid DC) lack TLR3. Immature DC in the periphery have a high capacity for endocytosis and antigen uptake. These immature cells are activated by and undergo maturation in response to microbial components. Engagement of TLRs on DCs by TLR ligands (presence of infection) leads to upregulation of both major histocompatibility complex (MHC) and costimulatory molecules.¹¹⁷ Mature cells lose the capacity for endocytosis and migrate into adjacent lymph nodes and there interact with naïve T cells to initiate the adaptive immune response.¹¹⁷

TLRs in the Eye

TLR4 and coreceptor CD14 have been found to be expressed by a variety of ocular tissues and cells including corneal epithelial cells,¹⁰ corneal stromal fibroblasts,¹⁸ human ciliary body, human iris endothelial cells (TLR4 only),¹⁷ resident antigen presenting cells (APCs) in the normal human uvea,¹³ and retinal pigment epithelial (RPE) cells.¹¹⁸ In a murine model of river blindness in which soluble extracts of filarial nematodes were injected into the corneal stroma, it was demonstrated that the predominant inflammatory response in the cornea was due to species of endosymbiotic *Wolbachia* bacteria. In addition, the inflammatory response induced by these bacteria was dependent on expression of a functional TLR4 receptor on host cells.¹¹⁹ TLR2 has also been found in human conjunctival and corneal epithelial cells.^{14,16,20} TLR-2 has been shown to play an active role in the chronic ocular inflammatory response to *Staphylococcus aureus* in conjunctival epithelial cells.²⁰ Kumar et al.,¹²⁰ observed that human corneal epithelial cells respond to *S. aureus* infection and Pam3Cys, a synthetic ligand of TLR2-challenge in a TLR2-dependent manner, suggesting that TLR is an innate receptor for *S. aureus* and functions as a Gram-positive bacterial sensor in the cornea. These data are consistent with a recent report showing that Pam3Cys stimulates PMN recruitment to the corneal stroma in a TLR2-dependent manner.¹⁹ Further study revealed that TLR2 was located at the cell surface by cell surface biotinylation and the treatment of corneal epithelial cells with TLR2-neutralizing antibody resulted in a significant decrease in Pam₃Cys-induced hBD2 production as well as IL-6, IL-8, and TNF- α secretion. However, contradictory results have been reported by Ueta et al.,¹⁶ who showed that TLR2 is expressed intracellularly in corneal epithelium and that peptidoglycan fails to stimulate cytokine production above basal levels. The reason for this discrepancy between the two laboratories in TLR2 cellular localization and function is not clear. It should be mentioned that a recent study suggested that the commonly used, commercially available peptidoglycan (used by both Ueta et al. and Kumar et al.) is contaminated by lipoproteins and LTA, and peptidoglycan is not a ligand of TLR2.¹²¹ However, a more recent study showed that *S. aureus* PGN is a TLR2 activator.¹²² Nevertheless, further studies, such as downregulation of TLR2 in cells by siRNA silencing or dominant negative expression, are needed to clarify the role of TLR2 in the reorganization of Gram-positive bacteria by HCECs.

In the eye, corneal epithelial cells constitute the first line of defense against microbial pathogens; therefore, these cells must possess the ability to discriminate the presence of pathogenic bacteria. Despite studies showing that microbial infection activates the TLR/IL-1R signaling cascade,¹²³ resulting in the expression of various proinflammatory cytokines and chemokines, the direct role of TLR/IL-1R in eye infections caused by bacterial pathogens such as *P. aeruginosa* and *S. aureus* remains underexplored. However, in a recent report using gene array, TLR expression as well as coreceptors including, but not limited to, CD14, IL-1R antagonist, TLR-6, and IL-18R accessory protein were detected.¹²⁴ Among others, these were all significantly elevated at the mRNA level in susceptible B6 versus resistant BALB/c mice.¹²⁵ The role of TLR9 also was tested in resistant versus susceptible mouse groups by determination of the effects of TLR9 silencing using RNA interference (RNAi) its activation in *P. aeruginosa* keratitis.⁹⁸ TLR9 mRNA levels were six times higher in B6 versus BALB/c cornea at 1 day after infection, and B6 mice injected with CpG DNA exhibited an increase in corneal mRNA for TLR9, IL-1 β , MIP-2, and IFN- γ over the control. B6 mice treated with TLR9 versus control siRNA, showed decreases in corneal opacity, PMNs, IL-1 β , and MIP-2 protein. In addition, fewer corneas were perforated in these mice, but bacterial load was elevated over the control subjects. Thus, signaling through TLR9 appears important in *P. aeruginosa* keratitis, and silencing TLR9 signaling reduces the host inflammatory response, but also likely contributes to the decreased bacterial killing in the cornea. These data on TLRs suggest an important immunomodulatory role for TLRs that may influence early, as well as

later events that occur in the disease response resulting in the susceptible versus resistance phenotype.

Khatri et al.¹²⁶ examined the role of TLR in a sterile keratitis model using an LPS challenge. When C3H/HeJ (TLR4 point mutation) versus C3H/HeN (control) mice were treated with LPS from *P. aeruginosa*, a significant increase in stromal thickness and haze was seen in the cornea of control C3H/HeN mice, but not in TLR4 mutant C3H/HeJ mice, and the severity of disease coincided with PMN stromal infiltration. The use of TLR knockout mice has shown clearly that activation of these receptors using several agonists leads to development of keratitis.¹⁹ Activation of TLR3 and -9 stimulated PMN recruitment to the cornea in wild-type but not in knockout mice. In addition, it has been shown that the corneal epithelium has functional TLR2 and -9 and that TLR2, -4, and -9 signal through myeloid differentiation factor-88.¹⁹ Clearly, further work is needed to elucidate the role of members of the TLR/IL-1R superfamily in models of microbial versus sterile keratitis, the former potentially resulting in perforation of the cornea and the latter resolving within approximately 24 hours after challenge. Overall, these and other animal studies provide provocative clues as to the mechanisms operative in the abrogation of immune privilege by a bacterial pathogen such as *P. aeruginosa*.¹²⁷ The significance of these data, particularly their correlation with human disease, awaits resolution.

In vitro studies have also been initiated using freshly isolated normal human corneal cells. Song et al.¹⁰ provided initial study showing that human corneal cells (epithelial, stromal, and endothelial) are capable of expressing the functional LPS receptor complex proteins CD14 and TLR4. TLR5, a receptor for flagellin, was detected at the corneal cell surface of basal and wing, but not superficial, epithelial cells.¹² Furthermore, TLR5 recognizes *P. aeruginosa* flagellin, eliciting an epithelial response through activation of the NF- κ B signaling pathway and producing proinflammatory cytokines and chemokines such as IL-6 and -8. Evidence also has shown that β -defensins, small antimicrobial peptides of the innate immune system respond to microbial infection of mucosal tissues and skin.¹²⁸ Both proinflammatory cytokines (e.g., IL-1 β) and *P. aeruginosa* upregulate human β -defensin-2 (hBD2) expression in corneal and conjunctival epithelial cells.^{129,130} Furthermore, stimulation of human corneal epithelial cells with lipoproteins and exoproducts of *S. aureus* also induces the expression of hBD2 in TLR2-dependent manner and the expressed hBD2 may contribute to the antimicrobial activity observed in the conditioned medium of TLR2-activated cells.¹²⁰ It was found that corneal epithelium is unique in that it utilizes TLR2 to sense and respond to Gram-positive bacterial infection through recognition of lipoproteins, but not lipoteichoic acid,^{14,120} a Gram-positive endotoxin that resembles LPS in some respects.⁷⁹ Considering the possibility that these cells may also be unable to recognize LPS,¹⁶ the unresponsiveness of corneal epithelial cells to the most common bacterial virulent factors may represent a mechanism by which epithelial cells avoid unnecessary activation when in contact with the bacterial endotoxin. By recognizing other PAMPs (e.g., lipoproteins, flagellin, and CpG DNA), these cells can still function as a sensor for detection of bacterial infection. TLR5 was localized to basal and wing cell layers, but not on the apical surface, of the corneal epithelium, supporting the notion that TLRs are activated only when there is a breach of the squamous cell layer. This may present a more general mechanism for limited stimulation of intact corneal epithelium.¹³¹ Once activated, the epithelium may also function as the first-line defender that secretes cytokines and chemokines that recruit inflammatory cells and produces antimicrobial molecules that directly kill pathogens.¹²⁰

Are toll-like receptors truly integral to the innate response to bacterial infections of the eye? In an effort to identify new and essential components of all TLR signaling pathways, the research group led by Dr. Beutler in the Scripps Research Institute used the alkylating agent *N*-ethyl-*N*-nitrosourea to generate random germline mutagenesis in mice and screened for new innate immune phenotypes.¹³² Among the mutant mice generated, *oblivious* is a nonsense

mutation of CD36.¹³³ CD36 is a coreceptor for TLR2/TLR6 heteromer that recognizes the R-enantiomer of MALP-2 (a diacylated bacterial lipopeptide) and to lipoteichoic acid. *Oblivious* mice are hypersusceptible to *Staphylococcus aureus* infection and undergo development of spontaneous eye infection at the age of 6 months with significant colonization of the surface of the eye by Gram-positive bacteria. By 1 year of age, endophthalmitis is frequently observed in *oblivious* mice. Spontaneous eye and skin infections was also observed in SCD1-mutant mice.¹³⁴ SCD1 is an enzyme responsible for the biosynthesis of the monounsaturated fatty acids, mainly palmitoleate (C_{16:1}) and oleate (C_{18:1}), both of which are bactericidal against Gram-positive (but not Gram-negative) organisms in vitro.¹³⁵ Furthermore, the clearance of skin infections by *Streptococcus pyogenes* and *Staphylococcus aureus*, but not by Gram-negative bacteria, is impaired in the *Scd1*-deficient mice.¹³⁴ Since transcription of *Scd1* is strongly and specifically induced by TLR2 signaling, SCD1 is recognized as a downstream effector of TLR2 signaling.¹³⁴ Taken together, these data suggest that a TLR-2-mediated innate response is essential for preventing Gram-positive bacterial infection in the eye. Although it is necessary for containing the infection, the host inflammatory response mediated by TLRs may also contribute to corneal destruction associated with bacterial keratitis, as it has recently been shown that activation of these receptors with specific ligands induces pronounced keratitis in mice.¹⁹

Herpes simplex keratitis is a disease initiated by infection of the epithelial and stromal layers of the cornea with HSV-1, resulting in infiltration of neutrophils and mononuclear lymphocytes into the stromal layer.^{136–141} Of interest in regard to viral infection, two TLRs, TLR3 and TLR9, have been shown to be expressed in the cornea.^{15,98,142} Stimulation of HCECs with the TLR3 agonist poly(I:C) induced the activation of NF- κ B and production of the proinflammatory cytokine IL-6 and chemokine IL-8. The expression of the antiviral cytokine IFN- β , the chemokine IP10, and the antiviral genes myxovirus resistance gene A and 2',5' oligoadenylate synthetase was also induced by poly(I:C).¹⁴² HSV-1 challenge of HCECs also resulted in the elevated expression of IL-6, IL-8, and IFN- β .¹⁴³ Of note, the expression of TLR7 was induced whereas the level of TLR3 was greatly downregulated.¹⁴³ Thus, in response to HSV-1 challenge, HCECs produce proinflammatory cytokines, leading to the infiltration and IFNs to enhance the antiviral activity in the cornea, probably through the action of TLR3, TLR7, and/or TLR9. In a mouse HSV-1 infection model, the inflammatory response to acute ocular HSV-1 infection was found to be associated with an increase in the secretion of chemokines, including IP10.¹⁴⁴ However, in TLR9 knockout mice, this HSV-1-induced IP-10 expression was hampered. Interestingly, TLR9-deficiency in C57BL/6 mice significantly increased virus shedding at the corneal surface whereas the total numbers of viruses in the cornea and in the trigeminal ganglion were not significantly affected (Carr D, University of Oklahoma Health Science Center, unpublished results, 2005). Thus, further study should reveal the role and the mechanism of action of TLR9 in the cornea in response to HSV-1 infection.

In addition to showing the function of TLR4 in innate immunity, a recent study attributed another important function to membrane-bound TLR4 in RPE cells.¹¹⁸ RPE mediates the recognition and clearance of effete photoreceptor outer segments (POS), a process central to the maintenance of normal vision. Exposure of human RPE cells to human, but not bovine POS, elicited transmembrane metabolic and calcium signals within RPE cells in a TLR4 dependent manner. Kinetic experiments of human POS binding to human RPE cells revealed that CD36 arrives at the POS-RPE interface followed by TLR4 accumulation within 2 minutes. Metabolic and calcium signals immediately follow. That (1) the bovine POS/human RPE combination did not elicit TLR4 redistribution, RPE signaling, or ROM release; (2) TLR4 arrives at the POS-RPE cell interface just before signaling; (3) TLR4 blockade with an inhibitory anti-TLR4 mAb inhibited TLR4 clustering, signaling, and ROM release in the human POS-human RPE system; and (4) TLR4 demonstrates similar clustering and signaling

responses to POS in confluent RPE monolayers suggests that TLR4 of RPE cells participates in transmembrane-signaling events that contribute to the management of human POS.¹¹⁸

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Figure 1.

TLR signaling and the role of epithelium in corneal innate defense. When cells are exposed to pathogens, TLRs such as TLR2, -4, and -5 at the cell surface recognize PAMPs such as lipoproteins, LPS, and flagellin, respectively. In contrast, nucleic acids released from damaged infected tissues or cells or captured from multiplying bacteria or viruses are brought to and recognized by TLRs (TLR3, -7, -8, and -9) in endosomes. TLR–ligand interaction triggers the activation of TLR signaling pathways in an MyD88-dependent or MyD88-independent manner. The MyD88-dependent pathway (all TLRs except TLR3) utilizes MyD88, Mal (for TLR2 and -4) as adaptors and transduces signal through IRAK1, IRAK4, and TRAF6, leading to the activation of NF-κB, p38, and JNK pathways and the production of proinflammatory cytokines and antimicrobial peptides. In contrast, the MyD88-independent pathway uses TRIF as an adaptor and transduces signal through TBK1 and IRF3/7, leading to the expression of IFNs and the IFN-induced genes.